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ODORS AND ORNAMENTS IN CRESTED AUKLETS (*AETHIA CRISTATELLA*): SIGNALS OF MATE QUALITY ?

A

DISSERTATION

Presented to the Faculty

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By

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Fairbanks, Alaska

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ODORS AND ORNAMENTS IN CRESTED AUKLETS (*AETHIA CRISTATELLA*):

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Abstract

Crested auklets (*Aethia cristatella*) are small colonial seabirds that display an ornamental feather crest and emit a citrus-like odorant during the breeding season. In this study odors and ornaments were investigated as possible signals of mate quality. Crest size was negatively correlated with the stress hormone corticosterone in males, but this was not the case in females. Body condition was negatively correlated with corticosterone in females, but this was not the case in males. Corticosterone levels were interpreted as an index of physiological condition, and it was concluded that males with longer crests were more competent at meeting the social and energetic costs of reproduction. I hypothesized that the crested auklet odorant: 1) functions as a chemical defense against ectoparasites, 2) is assessed as a basis for mate selection, 3) is facilitated by steroid sex hormones. Laboratory and field experiments showed that synthetic replicas of the crested auklet odorant repelled, impaired, and killed ectoparasites in a dose-dependent fashion. Chemical concentrations in plumage were at least sufficient to repel and impair ectoparasites. Chemical emissions from breeding adult crested auklets peaked at the time of egg hatching when young are most vulnerable to tick parasitism. In males, chemical emissions were correlated with crest size, a basis for mate selection. Presentation of synthetic aldehydes elicited behaviors similar to those that occur during courtship. Captive crested auklets responded preferentially to synthetic replicas of their odor, and the highest frequency of response occurred during early courtship. These results show that the chemical odor could be a basis for mutual mate selection. Production of the chemical odorant may be facilitated by steroid sex hormones since octanal emission rates

were correlated with progesterone in males. Finally it was determined that the chemical composition of odorants in crested auklets and whiskered auklets (*A. pygmaea*) differed in three key respects. This suggests that an evolutionary divergence occurred in the odorants of the two species similar to what has been suggested for ornamental traits. In conclusion, crested auklets appear to communicate with odors and ornaments, and these signals may convey multiple messages regarding condition, quality, and resistance to parasites.

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Preface

My curiosity about the citrus-like odor of the crested auklet (*Aethia cristatella*) was motivated by earlier experience with this species in the western Aleutian Islands of Alaska. I became interested in studying the crested auklet odorant while I was completing a master's degree in biology at Wake Forest University. Many individuals and organizations helped supported this research effort in addition to my dissertation committee. Specific acknowledgments are found in each chapter, but additional acknowledgments are provided here. Dr. William E. Conner of Wake Forest University encouraged me to persevere in this research, and his assistance with collaborations helped this work get started. Dr. Tappey H. Jones and Jason Co of Virginia Military Institute put a lot of effort into determining the chemical composition of the crested auklet odorant. The chemistry department at the Virginia Military Institute helped fund my visit to work with Tappey Jones on their campus. Dr. Julian Baumel helped me with dissections at the Burke Museum on the University of Washington campus. Drs. Gopinathan and Jaishri Menon were gracious hosts who helped me with histology and the scientific literature on avian epidermis.

Funding to conduct this research was provided by the Eppley Foundation for Research, Inc.; the Angus Gavin Memorial Bird Research Fund, University of Alaska Foundation; Society for Integrative and Comparative Biology Grant-in-Aid of Research; the David Burnett Dunn Memorial Award, University of Alaska Foundation; and the Howard Feder and David Shaw Graduate Student Support Fund. Logistical support that helped with the conduct of this research was provided by the Center for Global Change

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Native Corporation, City of Savoonga, Native Village of Diomede, Inalek Native Corporation, Ronald Ozenna, Jr. Jeff Williams obtained samples and specimens over the course of this research, and this has been an invaluable service. Other individuals provided assistance prior to the start of my dissertation research including Drs. Angela Glisan King and Bruce King of Wake Forest and Drs. James Barborak and Julian Lombardi of University of North Carolina Greensboro.

Introduction

The central hypothesis of this dissertation is that odors and ornaments in crested auklets (*Aethia cristatella*) are signals of mate quality. The signal intensity of ornaments and odors were compared to indices of quality in this colonial seabird. The crested auklet is a small, sooty-gray seabird (18-20 cm overall body length, mean adult mass 260 g, Jones 1993). The breeding range extends from the Diomede Is. south to the islands of the Aleutian-Shumagin Arc and west to the Russian Far East including Chukotka Peninsula, the Kurile Is., Sea of Okhotsk, and Sakhalin I. (Jones 1993, Springer et al. 1993). Crested auklets nest in large colonies located in cliff crevices, in the weathered rubble of old lava fields, and under rock talus and boulders formed by rockslides. The largest auklet colonies number in the hundreds of thousands of individuals (Springer et al. 1993). The crested auklet is named for a bushy crest of feathers that project from the forehead. A long ornate crest is produced in spring and displayed during the breeding season. This is complemented by a horny orange bill and white ornamental facial plumes extending behind the eye (Jones et al. 2000). The size of the crest is correlated with dominance (Jones and Hunter 1999), and both males and females tend to pair with mates that have larger crest ornaments (Jones and Hunter 1993, 1999). Evolution of the crest ornament may have been favored because it made the head and sharply hooked bill of males appear larger and more threatening to opponents (Douglas et al. 2005). During the breeding season the crested auklet produces a citrus-like odor detectable at considerable distances from colonies and aggregations at sea (Humphrey 1958, Jones 1993).

In vertebrates secondary sexual traits such as bright colors, extravagant plumage ornaments, and elaborate behavioral displays are theorized to provide information about the quality or condition of prospective mates (Fisher 1915, Williams 1966). Mate choice has direct effects upon the fitness of offspring through acquisition of genetic material, provision of parental care, and avoidance of parasites (Møller et al. 1999). Nevertheless, the significance of extravagant traits has been questioned since the origin of natural selection theory. Extravagant traits might be arbitrary or honest indicators. Darwin (1859) and Fisher (1930) each suggested that the exaggerated phenotypes of male animals are arbitrary bases for mate choice. Alternatively, proponents of viability models of sexual selection have suggested that extravagant male phenotypes, such as elaborate behaviors and plumage ornaments, can be honest advertisements of intrinsic quality or condition (Folstad and Karter 1992, Hamilton and Zuk 1982, Zahavi 1975, Zuk 1992). In this dissertation I sought to determine whether ornamental traits in crested auklets are honest or arbitrary signals.

Signals used in animal communication have been classified as conventional signals, assessment signals, or handicap signals, depending upon the costs for senders and receivers (Maynard Smith and Harper 1988, Dawkins and Guilford 1991, Vehrencamp 2000). Conventional signals may be arbitrary in their form, and they are not necessarily "honest" (Dawkins and Guilford 1991, Guilford and Dawkins 1995). Conventional signaling systems are vulnerable to invasion by "cheaters" because the signals indicate categorical membership rather than signaler quality (Dawkins and Guilford 1991). A principle cost that can help to maintain "honesty" in conventional signaling is retaliation

by receivers (Johnstone and Norris 1993, Vehrencamp 2000). Assessment signals have also been termed index, indicator, or condition-dependent signals because the degree of expression is limited by physiological or physical constraints (Vehrencamp 2000). Handicap signals tend to be correlated in their degree of expression with the sender's quality or the degree of risk or cost associated with producing the signal (Vehrencamp 2000).

Many birds are reported to have odors, and it has been suggested that these odors may be indicative of chemical defenses (Weldon and Rappole 1997). Odors may be derived from the actual chemical defenses, as argued in this dissertation for crested auklets. Alternatively, odors may be olfactory warnings of poison, analogous to aposematic coloration. This has been suggested for the hooded pitohui (*Pitohui dichrous*) and variable pitohui (*P. kirhocephalus*) of Papua New Guinea (Dumbacher et al. 1992). Both species are brightly colored and emit a sour odor (Dumbacher et al. 1992). The known poisonous agents in pitohuis belong to a class of potent nerve toxins called batrachotoxins (Dumbacher et al. 1992, 2000, 2004). It is unlikely that the batrachotoxins are odorants because they are relatively inert and have high molecular mass. Instead, the sour odor may be a different class of chemical compounds that function mainly as an olfactory warning.

I investigated the citrus-like odorant of the crested auklet with some basic principles of chemical ecology in mind (reviewed in Berenbaum 1995). Chemical compounds that are utilized in chemical defense are usually derived from primary metabolites, and as such they are secondary compounds not normally utilized in

metabolism. These defensive compounds are usually compartmentalized within specialized tissues to avoid the possibility of self-poisoning, and, therefore, there is often a method for delivery, discharge or activation of the chemical defenses. The optimal chemical defense should maximize the benefits of protection against natural enemies, but minimize the costs to the organism that deploys the chemical defense.

Chemical scents in vertebrates may also be acquired through natural or sexual selection for reasons unrelated to chemical defense (Darwin 1871; Blaustein 1981; Gorman and Trowbridge 1989). For example, chemical communication has an important role in reproduction of vertebrates (reviewed in Wyatt 2003). This might be true for crested auklets. Conspicuous sexual and social behaviors suggest that the auklet odorant is important in courtship (Hunter and Jones 1999; Douglas et al. 2001; Hagelin et al. 2003; Jones et al. 2004). Thus, avian plumage odors like the crested auklet odorant might be a form of chemical adornment rather than a means of chemical defense.

My research on chemical defenses in crested auklets was directed with several questions in mind. First, do the chemical compounds have a deleterious effect against ectoparasites? Second, do the chemical compounds occur in crested auklets at sufficient concentrations to interfere with parasitism? Third, what benefits are obtained from the chemical defense? Fourth, is this system consistent with expectations for chemical defense in terms of behavior, morphology, and ecology?

Each of the eight chapters in this dissertation relates to some aspect of odors and ornaments in crested auklets with respect to chemical ecology, endocrinology, behavior, and evolutionary biology. Chapter 1 reports chemical composition of the crested auklet's

odorant and proposes the ideas that were explored in this dissertation. This chapter was published in 2001 during the first year of work on this dissertation. Chapter 2 clarifies chemistry of the crested auklet odorant and reports on results of bioassays with ticks and lice. This chapter shows that the crested auklet and the closely related whiskered auklet differ in the chemical composition of their plumage odor. Bioassays show that the crested auklet odorant repels ticks in a dose-dependent fashion. Chapter 3 reports on the results of survival experiments with avian lice, and comparisons of louse abundances on least and crested auklets. The fieldwork carried out in this study helped to determine a new species of avian chewing louse (*Saemundssonia boschi*, Phthiraptera: Philopteridae) and contributed to revision of the louse genus *Saemundssonia* (Price et al., 2003). Survival experiments conducted with pigeon lice showed no effects on survival for crested auklet treatments. Ectoparasite loads were also compared between crested and least auklets, and crested auklets had more abundant lice. Chapter 4 shows that constituents of the crested auklet odorant are repellent to mosquitoes, and the results were comparable to similar experiments with commercial mosquito repellents.

Chapter 5 describes a novel research application for measuring chemical emission rates in crested auklets. Males and females did not differ in their mean octanal emission rates, but there was a sevenfold range among individuals. Chemical emission rates were compared to ectoparasite abundance. Tick abundance was very high in the auklet colony, but the infection rate on crested auklets was very low. The prevalence of louse infection was also very low. In Chapter 6, corticosterone levels were compared to ornamental traits. The prediction was that corticosterone would be negatively correlated with

ornament size, and this was confirmed for male crested auklets but not for females. Males and females also exhibited a different pattern with respect to body condition. Corticosterone was negatively correlated with mass:dimensional ratios in females but not in males. Chapter 7 describes seasonal patterns of chemical emissions and steroid hormone secretions in crested auklets. Results suggested a correspondence in the seasonal patterns of progesterone secretions and chemical emissions. Octanal was correlated with progesterone in males. There was a seasonal dynamic in progesterone, but this was not evident in other steroid hormones. Octanal emissions peaked at the time of egg hatching and early chick rearing. Progesterone also increased at the time of egg hatching and early chick rearing. It is suggested that progesterone may influence parental care. In Chapter 8, the chemical and behavioral evidence for mutual anointment are discussed. Behavioral experiments with a naïve captive population showed that crested auklets responded preferentially to taxidermic models that emit synthetic crested auklet odorant. Behavioral response peaked during early courtship, and this response was stronger when the chemical signal was stronger. A similar experiment with wild crested auklets showed that crested auklets perform anointment behaviors in response to synthetic replicas of *Aethia* odorants. Chapter 8 reports on specialized structures that secrete the crested auklet's chemical odorant to plumage, and it also shows that naturally occurring concentrations of aldehydes in plumage are sufficient to interfere with tick locomotion. All chapters in this dissertation have been published or submitted for publication in peer-reviewed scientific journals.

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Chapter 1: Heteropteran chemical repellents identified in the citrus odor of a seabird (crested auklet: *Aethia cristatella*): evolutionary convergence in chemical ecology[1]

Abstract The exogenous application of chemical repellents is widespread in birds, but endogenous production is exceedingly rare. We herein report a new class of avian defensive compounds isolated from the feathers and volatile odor of the crested auklet (*Aethia cristatella*). Mass spectra indicate that n-hexanal, n-octanal, n-decanal, Z-4-decenal and a 12-carbon unsaturated aldehyde comprise the auklet odorant. Octanal and hexanal are also secreted in the repugnant metasternal gland emissions of heteropteran insects and are known to be potent invertebrate repellents. We suggest that the auklet odorant functions as an ectoparasite repellent and a signal of mate quality. This would represent a rare and direct link between vigor, quality and parasite resistance, one of several putative bases for mate selection. This is the first report of defensive compounds produced by a seabird or colonial bird and one of the few examples of chemical defense in a polar or subpolar marine vertebrate.

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Introduction

Many birds apply naturally occurring repellents to their plumage or nests, arguably as a defense against ectoparasites, bacteria or fungi (Hart 1997). Experimental data may be inconclusive (Hart 1997), but the repellent properties of the chemicals used are well documented. For example, many passerine birds rub acid-secreting ants in their plumage while others apply marigold flowers and citrus peel (Clayton and Vernon 1993, Ehrlich et al. 1986). Marigolds contain the insecticide pyrethrum. Citrus peel contains limonene, a chemical that is toxic to a wide variety of arthropods; peel extract kills avian lice within seconds (Clayton and Vernon 1993). While the exogenous use of defensive materials has been recorded for more than 200 species of birds (Clayton and Vernon 1993), the endogenous production of defensive compounds appears to be rare. The best known examples are the nerve and muscle toxins (batrachotoxins) isolated from feathers and tissues of *Ifrita kowaldi* and the *Pitohui* birds of New Guinea (Dumbacher et al. 1992, 2000).

Two species of *Aethia* auklets, small planktivorous seabirds of the subarctic Pacific and Bering Sea, emit a pungent citrus odor (Jones 1993). We herein report that the compounds responsible for the citrus odor in the crested auklet (*A. cristatella*) are saturated and mono-unsaturated short-chained aldehydes. Such compounds are potent invertebrate repellents, and we suggest that the citrus odorant's primary function is ectoparasite repellency. This would be highly adaptive for colonial seabirds, and particularly *Aethia* auklets, which often nest in very large colonies (10^3–10^6 individuals; Springer et al. 1993). Increased parasitism is an expected cost of sociality (Duffy 1991;

Loye and Carroll 1995), and parasites probably limit group size in their hosts (Møller et al. 1993). Many seabirds are colonial, nest in large numbers at high densities, are long-lived, and return to the same breeding sites year after year (Duffy 1991). Seabird colonies thus represent large and dependable host populations. Ectoparasite infestations at these colonies can increase the cost of reproduction, increase mortality, introduce disease, reduce fecundity and growth rates, and contribute to the mass abandonment of colonies (Duffy 1991; Feare 1976; Loye and Carroll 1995; Møller et al. 1993).

Methods and Results

Crested auklet specimens were collected at Kiska, Aleutian Islands, on 26 May 2000 and shipped frozen to our chemical laboratories. We analyzed chemical constituents of the auklet's odor by headspace analysis and solvent extraction of feathers. We collected volatiles from two specimens as they thawed using a solid phase microextraction (SPME) fiber (1 cm in length) coated with 65 μm of polydimethyl siloxane/divinylbenzene partially crosslinked (Supelco). We placed each specimen in a 2,000 ml glass beaker covered with aluminum foil. The fiber was positioned near the specimen's neck, and samples were collected for 4.5 h. Mass spectra were obtained in the EI mode from a Shimadazu QP-5000 GC/MS equipped with a Rtx-5 (30 m × 0.25 mm) column. The injector and detector temperatures were held at 260°C throughout, and the column flow was 1.2 ml/min. The oven temperature was held at 60°C for the initial 3 min, then increased at a rate of 10.0°C/min to 250°C and held there for the remainder of the analysis. A standard pressure program was used. The SPME fiber was inserted directly into the injection port, and any adsorbents were allowed to volatize for the initial

5 min of the GC program. Mass spectra of the volatiles desorbed from the extraction fiber indicated the presence of nearly pure n-octanal (91.6%) and small amounts of n-decanal and Z-4-decenal. We identified the saturated aldehydes by direct comparison to known standards. Feathers clipped from the neck region of frozen birds were gently macerated and extracted in 1 ml of either methylene chloride or methanol (Fig. 1.1; bird 1 – methylene chloride, birds 2 and 3 – methanol) for 2 min. Mass spectra of the extracts indicated the presence of n-octanal (40.7%), n-decanal (3.4%), Z-4-decenal (8.2%), n-hexanal (21.4%), hexanoic acid (7.7%), octanoic acid (3.5%), and a 12-carbon unsaturated aldehyde (15.0%) not yet fully characterized. Large amounts of long-chained alcohols (16, 18, and 20 carbon) expected in the feather oils of seabirds (Jacob and Ziswiler 1982) were also present. The carboxylic acids associated with the feathers are common air oxidation products of the corresponding aldehydes. The appearance of hexanal in the extracts yet not in the headspace analysis is a reflection of the fact that the SPME fiber does not adsorb hexanal efficiently. Z-4-Decenal was identified from the mass spectra of its dimethylhydrazone (Attygalle et al. 1989, 1998), and by direct GC/MS comparison with a known standard (Lancaster Synthesis). We are conducting experiments to determine the structure and geometry of the dodecenal, and the results will be reported elsewhere.

Discussion

Octanal and hexanal are clearly odor components of the crested auklet's natural aroma. These compounds are frequently found in the repugnant metasternal gland emissions of the heteropteran insects, commonly known as stink bugs and squash bugs

(Blum 1981). Heteropteran secretions containing octanal and hexanal are potent invertebrate repellents (Aldrich 1988). We suggest that the octanal and hexanal in crested auklet feathers serve a similar function and repel invertebrate ectoparasites from adults and fumigate the nest site. Whiskered auklets (*Aethia pygmaea*) share the same citrus odor (Jones 1993), and we expect that the odorants serve the same function in that species.

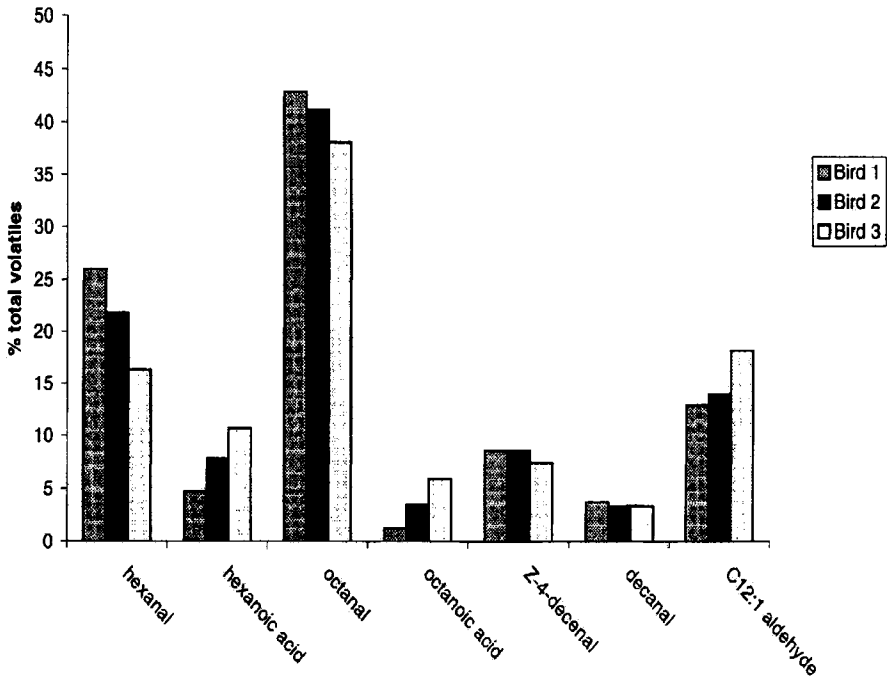

Fig. 1.1 Chemical composition of the crested auklet (*Aethia cristatella*) odorant. Histogram shows the relative abundances of each compound in the feather samples from three adult birds.

Hunter and Jones (1999) proposed that the crested auklet's citrus-like odor may be a sexually-selected trait, and they noted that it may be involved in a courtship display that they termed "ruff-sniff". We suggest that crested auklet's odorant could be involved in mate assessment, and it is perhaps analogous to the use of volatile green plant materials by starlings (*Sturnus vulgaris*). Incorporation of volatile green plants by male starlings into nests may attract females, impair the development of mites and bacteria, and improve vigor of nestlings (Clark and Mason 1988, Gwinner et al. 2000). Similarly, chemical potency may represent mate quality for crested auklets. During courtship, prospective mates bury their bills in each other's nape and neck feathers (Jones 1993, Hunter and Jones 1999). A mutual intertwining of necks (neck-twist display) occurs at advanced stages of courtship (Hunter and Jones 1999). Jones (1993) and (Hunter and Jones 1999) indicated that the odor is quite noticeable on the nape and neck feathers, and these displays appear to promote mutual odor assessment. This may help to explain why crested auklets and the closely related whiskered auklet complete courtship and copulation on the water (Hunter and Jones 1999). Auklets are likely to make a more accurate assessment of a potential mate's chemical production away from the dense, odor-saturated colony.

The advertisement of chemical potency and related ectoparasite resistance may be a basis for other extravagant traits in crested and whiskered auklets. Darwin (1871) suggested that odor glands are acquired through sexual selection, and Blaustein (1981: p. p. 1007) stated that "sexual selection should act upon these odours just as it acts upon visually conspicuous characters." The conspicuous forehead crests of crested auklets are

a focal point of courtship (Jones and Hunter 1993, Hunter and Jones 1999). Both sexes compete aggressively for mates with longer feather crests, an example of mutual sexual selection (Jones and Hunter 1993, Jones and Hunter 1999). Such extravagant traits in birds may act as advertisements of parasite resistance (Hamilton and Zuk 1982; Zuk 1991) and have been linked to immunocompetence in some species (Møller et al. 1999). We propose that odor production and ornaments are related secondary sexual traits in crested and whiskered auklets. Furthermore, the auklet's odor is both a signal of mate quality and functional in parasite resistance, a putative basis for mate choice. This potentially establishes a direct link between vigor, quality and parasite loads. Jones et al. (2000) found only weak correlations between crested auklet ornaments and body condition and concluded that the ornaments are not strong signals of quality. If these ornaments are cheap to produce, then the ornamental system of status signaling is vulnerable to cheating (Jones and Hunter 1999). Courting auklets then may look beyond ornaments to evaluate chemical production directly as an honest signal of mate quality (Zahavi 1975). The selective value of the citrus odor as an ectoparasite repellent and a mate attractant may have also spawned an indirect tertiary benefit. Individuals may orient to the citrus odor and thereby locate groups of conspecifics that have aggregated for the purpose of foraging.

The ultimate source of the auklets' citrus odor is presently unknown, though neither crested auklets nor whiskered auklets emit the odor in captivity at the Cincinnati Zoo (D. Oehler, Curator Cincinnati Zoo, pers. comm.). The aldehydes or their precursors may be sequestered from the auklets' natural food, which consists primarily of

zooplankton (Jones 1993; Springer et al. 1993). Aldehydes with even-numbered carbon chains comprise the auklet's citrus odor; this suggests biosynthesis by fatty acid metabolism. Isolation of these same aldehydes from a gland-like structure associated with the integument of crested auklets helps to support this contention (H.D.D., unpublished data).

The presence of specific aldehydes in both heteropteran insects and auklets suggests an evolutionary convergence on a common chemical solution to defense against invertebrates. It is reminiscent of the convergence of frogs and *Pitohui* birds upon batrachotoxins. Such evolutionary events help to focus attention on the nature and efficacy of particular chemical defenses. Chemical defenses are widely distributed among organisms at lower trophic levels where they function primarily as deterrents against predation (Berenbaum 1995). At upper trophic levels defensive compounds are rarer and, when they do exist, they may be more important as deterrents against parasitism (Dumbacher 1999; Mouritsen and Madsen 1994). Our findings represent a new class of avian defensive compounds. This is the first report of defensive compounds produced by seabirds or colonial birds and one of the few examples of chemical defense in a polar or subpolar marine vertebrate (Amsler et al. 2001).

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Chapter 2: Interspecific differences in *Aethia* auklet odorants and evidence for chemical defense against ectoparasites[1]

Abstract—The true auklets (*Genus Aethia*) are small planktivorous seabirds of the Bering Sea and North Pacific. Two species, the crested and whiskered auklets, produce volatile citrus-like odorants. We here show that the whiskered auklet odorant is composed predominantly of two odd-numbered aldehydes (heptanal and nonanal) with no detectable unsaturated aldehydes. By comparison, the crested auklet odorant is dominated by even-numbered aldehydes, both saturated and monounsaturated, ranging in size from 6 to 12 carbons. This is evidence of species-specific acquisition or biosynthetic pathways. We clarify the chemistry of the crested auklet odorant. We cite evidence that the C-12:1 aldehyde in crested auklets is actually two isomers, (*Z*)-4-dodecenal and (*Z*)-6-dodecenal. We also report on experimental evidence that aldehyde constituents kill and repel ectoparasites. Efficacy of the aldehydes may increase when they are combined in a mixture. The repellency of the mixture increases with chemical concentration. This suggests that individuals with higher chemical production are likely to repel ectoparasites more effectively.

Key Words—Crested auklet, whiskered auklet, aldehydes, (Z)-4-dodecenal, (Z)-6-dodecenal, synthesis, chemical defense, ectoparasites, pheromones, seabirds.

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INTRODUCTION

Aethia auklets are some of the most locally abundant avifauna in the Bering Sea, with colony sizes ranging up to 10^7 individuals (Springer et al., 1993). They dive at sea for their prey, and three of the four species feed almost exclusively upon zooplankton. Of these, the crested and whiskered auklet (*Aethia cristatella* and *A. pygmaea*, respectively) have an unusual citrus-like odor (Humphrey, 1958; Jones, 1993), while the least auklet (*A. pusilla*) does not have a noticeable plumage odor. The parakeet auklet (*A. psittacula*) has broad diet preferences that include invertebrates and fish, but a bill morphology that is probably best adapted to feeding upon gelatinous zooplankton (Harrison, 1990; Jones et al., 2001). The parakeet auklet also lacks the citrus-like odor (Jones et al., 2001; Hagelin et al., 2003). The phylogenetic relationships of the *Aethia* auklets have not been resolved using either morphological or molecular characters (Friesen et al., 1996). However, it seems plausible that crested and whiskered auklets are the most closely related due to similarities in their plumage ornaments, odorants, and vocalizations (Gaston and Jones, 1998).

In a previous report, we described the major components of the citrus-like odor of the crested auklet as a series of volatile aldehydes (Douglas et al., 2001; Fig 1.1). These are dominated by even-numbered, saturated and monounsaturated compounds, 6-12 carbons in length (hexanal, octanal, decanal, (Z)-4 decenal, and an unidentified C-12:1 aldehyde). We previously proposed that the odorant might be an avian chemical defense and a signal of mate quality (Douglas et al., 2001, 2002). Two major constitutents of the crested auklet's odorant, n-octanal and n-hexanal, are secreted in the metapleural glands

of stinkbugs and are potent invertebrate repellents (Aldrich, 1988). This suggests a convergence upon a common solution in defensive chemistry against invertebrate enemies (Douglas et al., 2001). Predation is the apparent selection pressure acting upon Heteropteran insects, whereas parasitism is the hypothetical selection pressure acting upon auklets. Indeed, parasitism appears to motivate exogenous anointment with defensive materials in many avian species. Many birds rub their plumage with materials that have chemical properties similar to the crested auklet's odorant. For example, birds crush ants in their bills and rub the secretions of ant metapleural glands through their plumage, a behavior known as "anting" (Ehrlich et al., 1986; Clayton and Vernon 1993). Similarly, the addition of green plant materials to bird nests has been discussed as a method of chemical defense against ectoparasites (Clark and Mason, 1985, 1988; Gwinner et al., 2000). Experimental evidence for the defensive efficacy of these behaviors remains elusive (Hart, 1997; Gwinner et al., 2000).

If the exogenous application of defensive chemicals is enigmatic, the endogenous production of chemical defenses is virtually unexplored. The pitohuis and *Ifrita kowaldi* of New Guinea are the few known examples (Dumbacher et al., 1992, 2000). These species produce or sequester batrachotoxins. These are potent nerve toxins with potentially broad defensive targets including both predators and avian lice (Dumbacher, 1999). Pitohuis also produce a sour odor that may signal their toxic properties (Dumbacher et al., 1992). Interestingly, odoriferous species are reported from eighty avian genera worldwide (Weldon and Rappole, 1997).

We herein clarify the chemistry of the crested auklet odorant, verify that its constituents repel or kill relevant ectoparasites in a dose-dependent fashion, and compare the odorants of crested and whiskered auklets.

METHODS AND MATERIALS

Birds. Crested Auklets. Six specimens were sacrificed and collected at Kiska I. (Lat. 52°06'N, Long. 177°36'E), Aleutian Islands, AK on 26 May 2000 and frozen soon afterwards. These specimens were shipped frozen to chemical laboratories at Virginia Military Institute and stored in an -80°C freezer. Analyses of odor components were conducted by headspace analysis and by extraction of the neck feathers. In the first method, volatiles were collected from two specimens as each thawed in a 2000 ml glass beaker covered with aluminum foil. Headspace analysis was conducted using a solid phase microextraction (SPME) fiber (1 cm in length) coated with 65 μm of polydimethyl siloxane/divinylbenzene partially crosslinked (Supelco Corp.). The fiber was positioned near the specimen's neck, and samples were collected for 4.5 hr and subsequently subjected to GC/MS analysis. For extraction, feathers clipped from the nape of three frozen birds were gently macerated and extracted in 1 ml of either methylene chloride or methanol for 2 min. The extract was analyzed by GC/MS. Subsequent analyses have shown that samples obtained from live birds with methanol feather extraction and headspace collection yield similar results.

Whiskered Auklets. Six specimens were sacrificed and collected near Tanager Pt., Chugul I. (Lat. 51°55'N, Long. 175°53' W), Aleutian Islands, AK on 4 June 2001 and frozen soon afterwards. These specimens were shipped frozen to the University of Alaska

Fairbanks and stored in a -20°C freezer. The nape feathers from six specimens were gently macerated and extracted in 1 ml of methanol for 2 min. The extracts were analyzed by GC/MS.

Chemical Analysis. Gas chromatography–mass spectrometry was carried out in the EI mode using a Shimadzu QP-5000 GC/MS equipped with a RTX-5, 30 m × 0.032 mm i.d. column. The instrument was programmed from 60 to 250°C at 10°/min. Vapor phase FT-IR spectra were obtained using an Hewlett-Packard model 5965B detector interfaced with an Hewlett-Packard 5890 gas chromatograph fitted with a 30 m × 0.25 mm RTX-5 Amine column. High Resolution mass spectrometry was performed on a JEOL SX102 instrument. Identification of the compounds was confirmed by direct comparison of their mass spectra and retention times with those of commercial or synthetic pure compounds.

Synthesis. It was necessary to synthesize standards to confirm identities of the 12 carbon aldehydes in the crested auklet odorant because no commercial standards are available.

(*Z*)-4-Dodecenal. A solution of sodium methylsulfinyl–methanide (16 mmol) was prepared from 400 mg of NaH in 5 ml of dimethylsulfoxide (DMSO) and combined with 15 ml of tetrahydrofuran (THF). Triphenyl-phosphonium bromide, prepared from 2-(3-bromopropyl)-1,3-dioxolane and triphenylphosphine, in the quantity of 3.6 g (7.9 mmol) was added to this mixture at 25°C under argon. After 10 min, the mixture was cooled to 0°C and treated with 1.2 ml of freshly distilled octanal. The mixture was stirred for an hour, poured into distilled water, and extracted with ether three times. The ether fraction

was dried under reduced pressure, and the residue was extracted several times with petroleum ether. After removal of the solvent, the mixture was heated to 80°C for 2 hr in 5 ml of concentrated acetic acid. The solvent was removed, and the residue was resuspended in ether, and neutralized with saturated $NaHCO_3$. After drying over $MgSO_4$, removal of the solvent provided 1.2 g of a mixture that was greater than 80% a single component, MS *m/z* (rel %) 164 (M-18, 2), 138(5), 111(3), 110(4), 109(3), 98(10), 97(12), 96(7), 84(72), 83(32), 82(10), 81(12), 79(10), 70(14), 69(15), 68(12), 67(23), 57(10), 56(13), 55(48), 54(30), 53(5), 43(67), 41(100). A sample of this product was suspended in petroleum ether and heated to 60°C with dimethylhydrazine for 4 hr. After the solvent was removed, the (Z)-4-dodecenal was obtained as a single major component, MS *m/z* (rel %) 224(M-18, 3), 209(1), 182(3), 180(1), 156(1), 139(2), 125(2), 111(1), 95(2), 86(9), 85(100), 69(3), 67(5), 55(7), 44(36).

(*Z*)-6-Dodecenal. A solution of sodium methylsulfinylmethanide was prepared from 600 mg of NaH in 15 ml of DMSO, and this was placed in 15 ml of THF. To this was added a solution containing 4.4 g (10 mmol) of triphenylphosphonium (6-hydroxyhexyl) bromide dissolved in 12 ml of DMSO under literature conditions (Horiike et al., 1978). After 20 min, 1 ml of freshly distilled hexanal was added, and the reaction was worked up in the prescribed manner. Half of the crude product was added to a slurry of pyridinium chlorochromate in CH_2Cl_2 (3 g/50 ml). This mixture was stirred for 3 hr. After the addition of 150 ml of ether, the mixture was filtered through a short florisil column. The solvent was removed to provide 0.5 g of an oil that was 80% a single component: MS *m/z* (rel %) 164 (M-18, 3), 135(2), 121(7), 98(12), 97(16), 96(9), 95(9),

94(5), 93(10), 84(16), 83(20), 82(18), 81(25), 80(9), 79(24), 70(24), 69(23), 68(10), 67(34), 57(22), 56(20), 55(84), 54(40), 53(10), 43(44), 41(100). A sample of this was suspended in petroleum ether and heated to 60°C with dimethylhydrazine for 4 hr. After the solvent was removed, the (Z)-6 dodecenal was obtained as a single major component: MS m/z (rel %) 224 (M-18, 3), 209(1), 195(1), 180(6), 167(1), 153(2), 142(9), 122(9), 99(8), 95(8), 86(40), 85(27), 73(15), 67(11), 60(30), 59(48), 45(42), 44(100), 43(50), 42(45), 41(49); HRMS, calculated for $C_{14}H_{28}N_2$: 224.2252: observed 224.2247.

Repellency Trials. We adopted a moving object bioassay for tick repellency tests from Dautel et al. (1999). A heated rotating drum emulated host cues of heat and motion. The drum (21.3 cm long × 11.5 cm diam.) full of water was maintained at 38°C with an aquarium heater (Slim-Tech #8814PC). A Pioneer turntable powered by a Dayton DC Motor (Model 1Z835) and regulated by a DC pulse-width modulated speed controller (Model CK 1400, Carl's Electronics, Sterling, MA) rotated the drum at 10.5 rpm. A raised plate (7.6 × 3.2 × 0.2 cm thick) on the drum's surface afforded a point of attachment where questing nymphs could attach to the artificial host per their natural behavior. These nymphs were placed on a metal rod (4 mm diam.), positioned perpendicular to the rotating drum with the end of the rod just 1.1 mm from the surface of the plate (after Dautel et al., 1999). The rod was sheathed in heat shrink tubing to improve the tick's ability to adhere. Filter paper (Whatman 2 Qualitative) was cut to fit the raised plate; chemicals were added to the filter paper until saturated (0.008–0.025 ml/cm^2). The paper was allowed to volatize 10 min prior to placing it on the raised plate. Volatile repellents wane in effectiveness after 1 hr (e.g., DEET; Dautel et al., 1999). If a

trial extended more than 1 hr, the filter paper was saturated again, allowed to volatize for 10 min, and then the trial was resumed.

During laboratory and field trials, individual nymphs were picked up with a fine-haired camel brush and placed 8 mm from the rod tip, oriented towards the rotating drum. Each tick was observed for 2 min, and the following data were recorded: approached drum (yes/no), time to walk to rod tip, time at tip, attached to drum (yes/no), and duration of attachment. We used nymphs of *Amblyomma americanum* for laboratory trials, and nymphs of *Ixodes uriae* (Acari: Ixodidae) for field trials. *Ixodes* ticks may occur in high densities at some colonies, and they parasitize auklets throughout most of their range in Alaska. *Amblyomma* ticks do not occur in the auklets' range; however, they do commonly parasitize birds and mammals. Nymphs of *Amblyomma* were obtained from Oklahoma State University. *Ixodes* ticks for field trials were obtained from a crested auklet colony above Yukon Harbor, Big Koniuji Island, AK. These *Ixodes* ticks were deposited in the U.S. National Tick Collection (curated at Georgia Southern University) under accession number RML 123386.

The repellency trials addressed two questions: (1) Do the dominant odorant constituents of crested auklets repel ticks in a dose dependent fashion? (2) Do different aldehyde constituents of crested auklets differ in their level of repellency? In order to answer these questions, we compared duration of attachment in two lab experiments (I and II) and a field experiment (III). The objective of Experiment I was to determine whether octanal, the chief ingredient in the crested auklet's odorant, is repellent to ticks. This research was conducted in the lab with a treatment of 10% octanal in ethanol, an

ethanol control, and a blank control. We also tested the ethanol control to determine whether it is more repellent than the blank filter paper. In Experiment II, the objective was to calculate a dose–response curve for repellency and determine the level of significant effect. A synthetic cocktail of the crested auklet odorant constituents (40% octanal, 21% hexanal, 8% (Z)-4 decenal, 3% decanal, 7% hexanoic acid, 3% octanoic acid) was presented at three concentrations (100%, 10% in ethanol, and 1% in ethanol). The percentages of aldehydes refer to relative abundance of each aldehyde per total volume of cocktail (the actual purity of each aldehyde in the auklet odorant is relatively high, e.g., >92% in the case of octanal; Douglas et al., 2001). These treatments were compared to an ethanol control. Dodecenals were not used in the cocktail because they are not commercially available. Experiment III was conducted at Big Koniuji Island in the Shumagin Islands, AK with three treatments: (1) octanal, (2) decanal, (3) aldehyde mixture (octanal 40%, hexanal 20%, decanal 3%. in ethanol). These treatments were compared to an ethanol control. The objective was to compare the repellency effect of crested auklet odorant constituents individually and in combination.

We used the following commercial synthetic chemicals for all bioassays: Hexanal 96% (C.A.S.: 66-25-1), Octanal 99% (C.A.S.: 124-13-0), Decanal 95% (C.A.S.: 11231-2), Hexanoic acid 99+% (C.A.S.: 142-62-1), Octanoic acid 99% (C.A.S.: 124-07-2) by ACROS Organics, and (Z)-4 Decenal 95% (C.A.S.: 21662-09-9) by Lancaster Synthesis. Laboratory trials were conducted at 22°C and at moderately high humidity. Field trials were conducted inside a tent with moderately high humidity and at ambient temperatures

that fluctuated between 14–22°C. Results of control and experimental trials were compared with an independent measures t-test, after evaluating homogeneity of variance.

Mortality Tests. Mortality trials were conducted in the field with nymphs and adults of *Ixodes uriae* at Big Koniuji I., AK. The objective was to determine whether small quantities of a crested auklet odorant constituent would cause mortality. We chose octanal because it is the most abundant odor constituent. Distilled water was used for the control. Each tick was placed in an individual glass vial (Fisher #03339-21F). Six adults and four nymphs were placed in the control. Seven adults and three nymphs were placed in the octanal treatment. We used a 100 μl Hamilton Syringe (Model 84859 810RN) to apply the smallest quantity of liquid possible (5 μl) to the tick without touching the syringe needle to the tick.

Mortality trials were also conducted with auklet lice obtained from crested auklets at Little Diomede Island, AK on August 28, 2002, using a carbon dioxide fumigant, according to methods reported in Visnak and Dumbacher (1999). Fifteen lice were used in each of three treatments for a total of 45 lice. In each treatment, we placed an individual louse on an individual crested auklet feather. Next 1 μl of substance (octanal, (Z)-4 decenal, or tap water with soap) was applied to the opposite side of the feather with a Hamilton Syringe. We used tap water with soap (1 drop/cup) as a control. Soap was added as a wetting agent since avian feather oils have hydrophobic properties. This was replicated 15 times for each treatment. Lice were checked at the time of application and then at 1 min, 1, and 5 hr elapsed time after application.

The design for the louse mortality experiment was identical in all respects. Feathers for this experiment were selected from a single crested auklet specimen in June at Big Koniuji Island. The feathers were first placed in a petri dish and left in sunlight. Light and exposure to oxygen break down the auklet aldehydes. We noticed that after 1 week, the remaining concentrations of aldehydes in feathers, if present, are below the threshold of human detection. After 1 month, we selected feathers of identical size, shape, and length; all feathers had similar surface areas. Five feathers were suspended by the calamus (equidistant from each other) from an aluminum foil lid. Next, the foil was firmly attached to the rim of a 500 ml glass jar. Three glass jars were prepared for each treatment, and this accommodated 15 lice for each treatment. To conduct the trials we removed the foil lid and lay it upside down with the feathers facing straight up. A louse was placed on each feather. The substance was applied to the feather. Then, the foil lid was secured back on the jar, and the lice were observed through the jar. These lice were deposited into the frozen collections of the Price Institute for Phthirapteran Research at University of Utah in 2004.

RESULTS

Chemical Analysis. Crested Auklets. Solid phase microextraction (SPME) from two birds provided the initial indication that the odor was due to the presence of short chain aldehydes (Douglas et al., 2001). GC/MS analysis of the absorbed volatiles revealed the presence of octanal, (*Z*)-4-decenal, and decanal, all confirmed by comparison with commercial samples (Douglas et al., 2001). Solvent extraction of the neck feathers from three birds provided a more complete and consistent analysis. GC/MS

analysis of the extract revealed a complex mixture of high molecular weight oils along with a number of volatile components. The latter contained hexanal (16–26%), octanal (38–43%), (*Z*)-4 decenal (7.4–8.6%), decanal (3–4%), and a C-12:1 aldehyde (13–18%): MS m/z (rel %) 164 (M-18, 2), 138(2), 135(2), 121(4), 98(9), 97(10), 96(5), 95(4), 84(22), 83(18), 82(11), 81(17), 80(7), 79(14), 70(14), 69(18), 68(10), 67(25), 57(20), 58(17), 55(61), 54(25), 43(43), 41(100). Our analyses did not find trace chemicals reported by Hagelin et al. (2003).

Initially, the C-12:1 aldehyde was assumed to be a (*Z*)-4 isomer, homologous with the (*Z*)-4-decenal present in the mixture. Closer inspection of the GC peak showed that the intense ion at m/z = 84, characteristic of (*Z*)-4-dodecenal, was not congruent with the total ion current for that peak, matching only the leading edge of the peak. When the peak was scanned from the beginning to the end, the ion at m/z = 84 changed in intensity from 72 to 16 relative %. Further examination of the spectra from the trailing edge of the peak suggested the presence of (*Z*)-6-dodecenal (Horiike and Hirano, 1988). These data indicated that the unidentified C-12:1 aldehyde was actually a mixture of two isomers.

Dimethylhydrazones of the extract were prepared, since the mass spectra of these derivatives have been used to assign double bond position in unsaturated aldehydes (Attygalle et al., 1989, 1998). The front edge of the peak for the dimethylhydrazone of the dodecenal (m/z = 224, M+) showed an intense ion at m/z = 85 (80%), and the trailing edge of the peak showed an ion at m/z = 142 (10%). Selected ion monitoring showed that these two ions were not congruent with each other or with the total ion current for this peak. The intense ion at m/z = 85 in these derivatives is indicative of a C-4 double bond,

while the ion at *m/z* = 142 results from the splitting out of cyclohexene that occurs in the mass spectra of these derivatives with a double bond at the C-6 position (Fig. 2.1). The assignment of both (*Z*)-4-dodecenal and (*Z*)-6-dodecenal was confirmed by direct comparison of the natural component with synthetic samples prepared as shown in Figure 2.2.

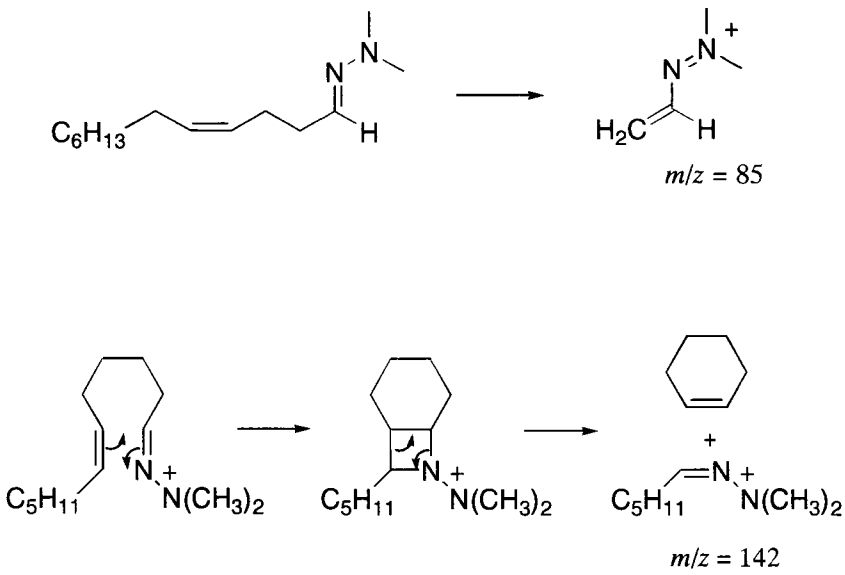

FIG. 2.1. Fragmentation ions from (*Z*)-4 and (*Z*)-6 dodecenals.

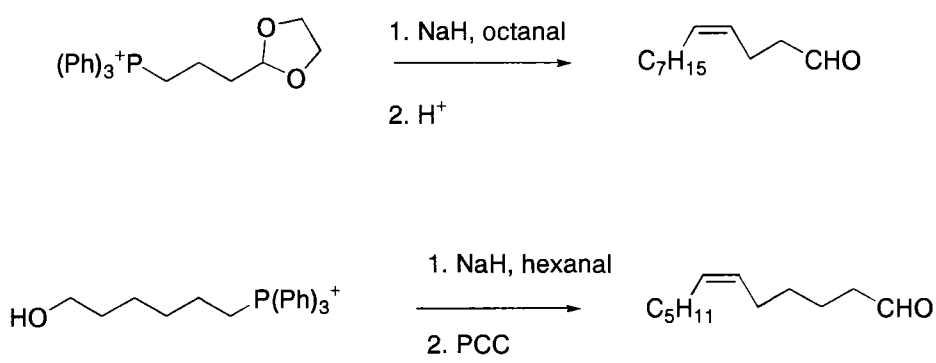

FIG. 2.2. Preparation of (*Z*)-4 and (*Z*)-6 dodecenals.

The Wittig conditions used in the syntheses produced small amounts of the E isomers (<5%) sufficient to permit the assignment of double bond geometry by comparison, and the natural material matched the major (*Z*) synthetic component in both cases. A mixture of the dimethylhydrazones from the synthetic (*Z*)-4-dodecenal and (*Z*)-6-dodecenal also had identical mass spectral properties and GC retention times to the chromatographic peak for the dimethylhydrazone of the C-12:1 aldehyde from the natural extract.

Whiskered Auklets. The chemical composition of crested auklet and whiskered auklet odorants is compared in Table 2.1. Analysis of whiskered auklet feather extracts revealed the presence of heptanal, nonanal, decanal, and hexadecanol in an average ratio of 2.3:2.3:1:4.7. The MS and GC retention times were identical to those of authentic samples.

Repellency Trials. Moving object bioassays demonstrated that the aldehyde odorant of crested auklets is repellent to ticks. Duration of attachment to the artificial host was significantly less for aldehyde treatments than for the ethanol and blank filter paper controls. In all three experiments (I-III), the majority of ticks (>93%) consistently walked to the end of the rod and many of these quested for the artificial host. However, they were repelled when the filter paper with aldehydes passed the end of the rod. In these instances, some nymphs returned to quest a second and third time but were repelled on each occasion. Some of those ticks that did attach to the aldehyde treated filter paper fell off soon afterwards.

TABLE 2.1. Percent composition of odorant in crested auklets (*A. cristatella*) compared to whiskered auklets (*A. pygmaea*) as determined from feather extracts.

Compounds	*A. cristatella*	*A. pygmaea*
Hexanal	21.4	—
Hexanoic acid	7.7	—
Heptanal	—	22.3
Octanal	40.7	—
Octanoic acid	3.5	—
Nonanal	—	22.3
Decanal	3.4	9.7
Z-4-Decenal	8.2	—
Z-4-Dodecenal	7.5	—
Z-6-Dodecenal	7.5	—
Hexadecanol	—	45.6

Experiment I. Nymphs of *A. americanum* were repelled by the octanal treatment (Figure 2.3). Duration of attachment was greater for the ethanol control (72.4 ± 8.8, sec, N = 30) than for the octanal treatment (27.7 ± 11 sec, N = 20), $t(48)_{1\text{-tailed}} = 3.16$, $P<0.005$. Duration of attachment was also greater for the blank paper control (62.7 ± 9.8 sec, N =30) than the octanal treatment, $t(48)_{1\text{-tailed}} = 2.33$, $P<0.05$.

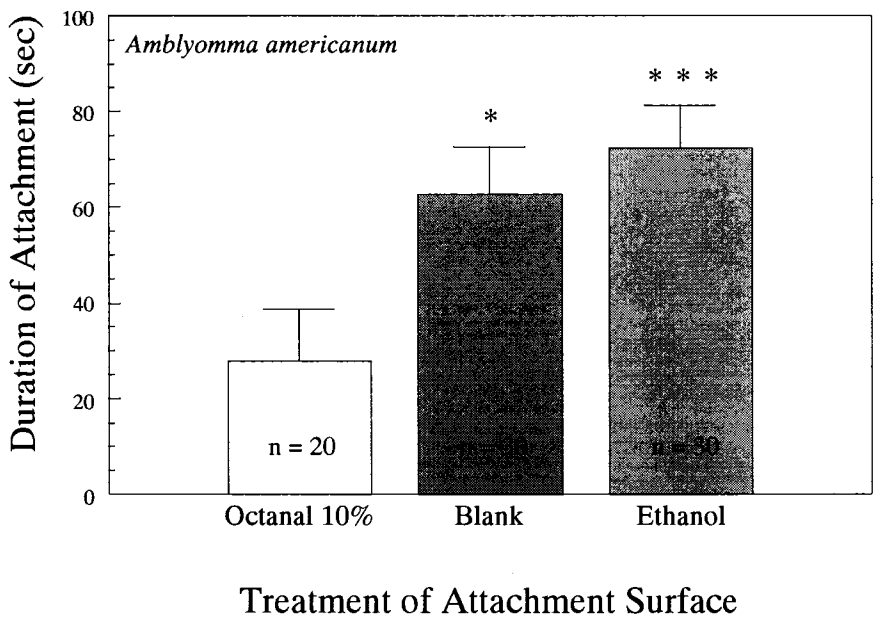

FIG. 2.3. Experiment I: Ticks were repelled in a moving object bioassay by filter paper treated with octanal. Nymphs of *Amblyomma americanum* were repelled by filter paper treated with 10% octanal in ethanol. Duration of attachment to the artificial host was less for the octanal treatment than for either control treatment (blank filter paper and ethanol treated filter paper). A single asterisk indicates significance at $P<0.05$. Three asterisks indicate significance at $P<0.005$.

Fewer ticks attached to the octanal treatment (25%) than the ethanol control (80%), $t(48)_{1\text{-tailed}} = 4.0$, $P<0.001$. The difference was also significant in comparison with blank filter paper; 63% of ticks attached to this control, $t(48)_{1\text{-tailed}} = 2.13$, $P<0.05$. The ethanol control and the blank control did not differ for duration of tick attachment ($t(58)$

= 0.73, $P_{1\text{-tailed}} = 0.24$). A higher percentage of ticks attached to the ethanol control (80%) compared to the blank control (63%), and this difference was just significant ($t(58) = 1.70$, $P_{1\text{-tailed}} = 0.047$). So ticks were not repelled by ethanol. Instead, ethanol may be a mild attractant.

Experiment II. Repellency of the aldehyde odorant increases with concentration (Figure. 2.4). The average duration of attachment did not differ between the 1% concentration (42.8 ± 10 sec) and the blank control (49.9 ± 9.7 sec), $t(57)_{1\text{-tailed}} = 0.399$, P=0.34. Increasing the aldehyde concentration to 10% reduced the average duration of tick attachment to 13.5 sec (±5.9), and this was less than the blank control $t(48)_{1\text{-tailed}} = 3.20$, P<0.005. At 100% concentration, the average duration of attachment was 0.3 sec (±0.5), significantly less than the blank control, $t(29)_{1\text{-tailed}} = 5.09$, P<0.001.

Experiment III. Constituents of the crested auklet aldehyde odorant were repellent to nymphs of *I. uriae*, an ectoparasite of crested auklets (Figure. 2.5). Duration of attachment for the ethanol control averaged 63.4 sec (±8.9) vs. 10.8 sec (±5.4) for octanal, 31.6 sec (±7.5) for decanal, and 24.3 sec (±7.1) for the aldehyde mixture. All of these differences were statistically significant: octanal, $t(58)_{1\text{-tailed}} = 4.85$, P<0.01; decanal, $t(58)_{1\text{-tailed}} = 2.06$, P<0.05; aldehyde mixture consisting of 40% octanal, 20% hexanal, 3% decanal in ethanol, $t(58)_{1\text{-tailed}} = 2.78$, P<0.01.

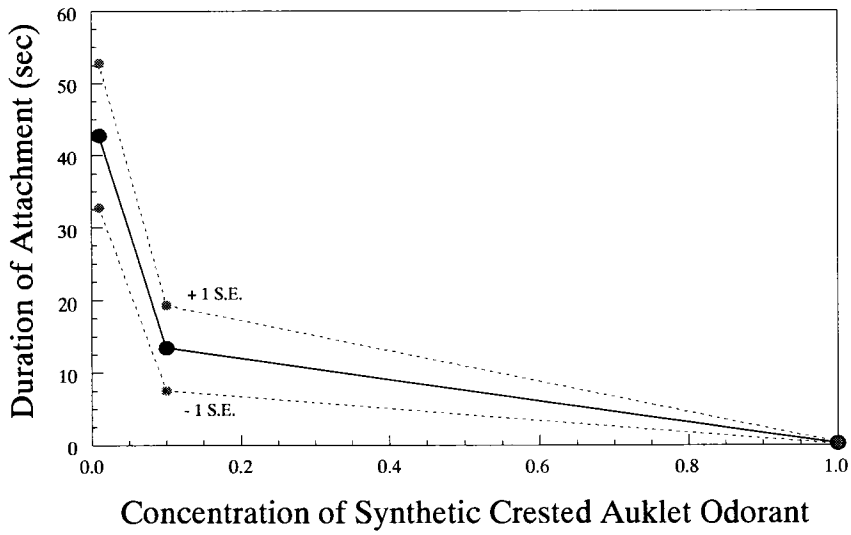

FIG. 2.4. Experiment II: Nymphs of *Amblyomma americanum* exhibit dose-related response to varying concentrations of synthetic crested auklet odorant in ethanol. Response is measured as duration of attachment (sec) to an artificial host. Repellence is higher at higher concentrations. Attachment time was greater for the control than the synthetic blend at 100% ($P<0.001$) and 10% ($P<0.005$) but not at 1% concentration ($P=0.34$).

Nymphs of *I. uriae* were more likely to attach to the ethanol control than the aldehyde treatments. Seventy percent of nymphs (N = 30) attached to the control. By comparison, only 20% of nymphs attached to octanal ($t(58)_{1\text{-tailed}}$ =-4.43, $P<0.001$); 43% attached to decanal ($t(58)_{1\text{-tailed}}$ =-2.13, $P<0.05$); and 40% attached to the aldehyde mixture ($t(58)_{1\text{-tailed}}$ =-2.41, $P<0.01$). Repellency of the dilute aldehyde mixture (63% concentration in

ethanol) was equivalent to nearly pure decanal (Figure. 2.5). Duration of attachment did not differ for the two treatments ($t(58)_{2\text{-tailed}} = 1.67$, $P > 0.1$).

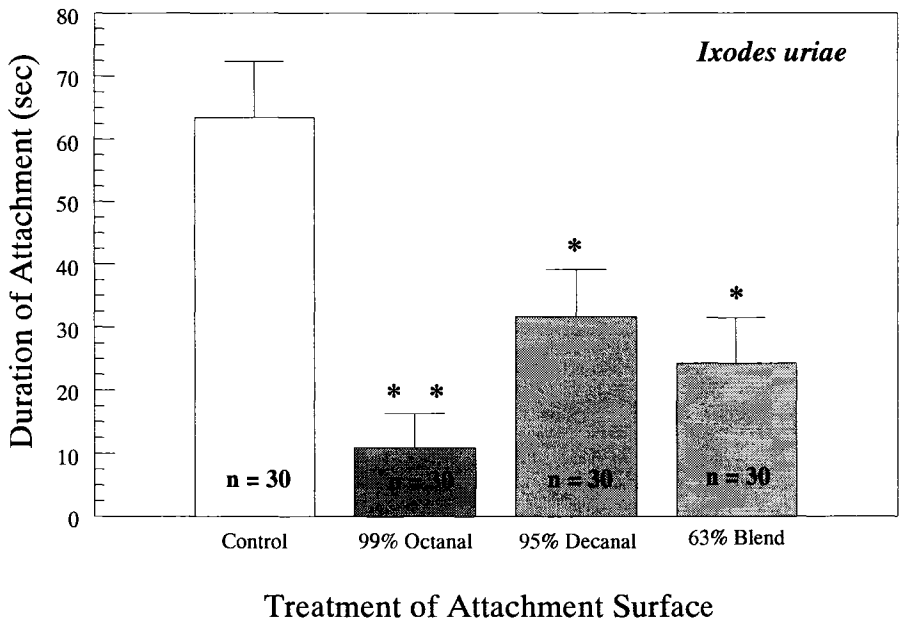

FIG. 2.5. Experiment III: Repellency of crested auklet odorant constituents to nymphs of *Ixodes uriae* ticks. A single asterisk indicates significance at $P<0.05$. Two asterisks indicate significance at $P<0.01$. The mixture of odor constituents at 63% concentration in ethanol was as repellent as nearly pure decanal.

Mortality Trials. Tick nymphs in the octanal treatment became moribund in less than 15 min, and tick adults did so in less than 1 hr. None of these ticks recovered within 2 days. All ticks in the control treatment were still active 2 days later. All lice in the

experimental treatments (octanal and (*Z*)-4-decenal) became moribund within seconds. All lice in the control treatment (tap water with soap) were active up to 12 hr later. The control and (*Z*)-4-decenal treatments included lice from the genera *Austromenopon* and *Quadraceps*. The octanal treatment only included lice from the genera *Austromenopon*.

DISCUSSION

Our combined studies suggest that the aldehyde odorants of crested and whiskered auklets are truly species-specific and not the result of differences in location, year of collection, or diet. We are continuing to investigate the intraspecific variation in crested and whiskered auklet odorants, but at present the variation within species is less than the variation between species. As previously noted, we have also isolated the same crested auklet odorant constituents from a gland-like structure (Douglas et al., 2001).

Our chemical analysis differs slightly from Hagelin et al. (2003). They reported higher relative abundances for two oxidation products—hexanoic acid and octanoic acid. This difference is likely due to air oxidation of the aldehydes (Loudon, 2002). Hagelin et al. (2003) also reported trace compounds not discussed in our report. Those differences may have been the result of different methods for odorant collection (Raguso and Pellmyr, 1998).

Aethia aldehyde odorants appear to function as ectoparasite repellents (Douglas et al., 2001). Constituents of the crested auklet odorant repel ectoparasites, and mixtures may have synergistic properties. In plumage these repellents may interfere with parasitism by ticks and lice. *Ixodes uriae* ticks occur in high densities at auklet colonies.

Ixodes ticks serve as vectors for pathogens such as Borrelia (Olsén et al., 1993), and infestations have been associated with increased mortality at some seabird colonies (Bergström et al., 1999). Chewing lice (Order Phthiraptera) are also abundant in some cases, and heavy louse infestations are known to reduce fitness in birds (Clayton, 1990). Louse abundance (Genera *Saemundssonia, Quadraceps, Austromenopon*) can exceed 200 individuals on some chicks and adults. Conversely, many adults within a population have few lice. For example, at Talan Island in 1988, lice were found in abundance only on crested auklet fledglings or chicks (11 infected, N = 33; E. Hoberg, U.S. National Parasite Collection). No lice were observed on 80 adult crested auklet males and females by careful visual inspection. Individuals with heavy louse infestations may serve as parasite reservoirs in avian populations, and chemical defense may serve to reduce louse transmission in highly social species like the crested auklet.

The crested auklet odorant may function as a signal of mate quality (Douglas et al., 2001). We have not noted sex-specific differences in chemistry of the auklet odorant, but we suspect that one important component of the signal is quantitative. Crested auklets that produce more of the aldehyde odorant are likely to repel ectoparasites more effectively in a dose-dependent fashion, and this would portend benefits for potential mates. Parasites harm hosts by diverting resources and acting as vectors of disease (Møller et al., 1999). Fitness benefits of parasite-free mates are lower susceptibility to disease and less likelihood of parasite transmission to mates and offspring (Loye and Carroll, 1995; Møller et al., 1999). Crested auklets may evaluate mate quality on the basis of chemical potency, and courtship behaviors could provide an opportunity for

assessment. During courtship, crested auklets bury their bills in the nape feathers of prospective mates—"ruff-sniff" behavior (Jones, 1993; Hunter and Jones, 1999; Hagelin et al., 2003). Preliminary evidence suggests that crested auklets orient to constituents of their odorant (Douglas et al., 2002; Hagelin et al., 2003) indicating that they may be capable of smelling the aldehydes they produce.

The *Aethia* aldehyde odorants offer clues to unraveling the complex phylogeny of the true auklets. Molecular evidence suggests that *Aethia* auklets speciated rapidly as part of an adaptive radiation, but the evidence falls short of clarifying the interspecific relationships within this genus (Friesen et al., 1996). Gaston and Jones (1998) suggested that crested and whiskered auklets are more closely related based upon shared traits. Our results support and elaborate upon this working hypothesis. Characteristics of the crested and whiskered auklet odorants suggest an evolutionary divergence. Both species produce decanal in their odorant. However, feather extracts of whiskered auklets differ from those of the crested auklet in three ways: (1) dominance of odd-numbered aldehydes; (2) lack of unsaturated aldehydes; (3) presence of hexadecanol. These differences suggest species-specific biosynthetic or sequestration pathways for the *Aethia* auklet odorants. The pattern of divergence in chemical odorants is paralleled by patterns of divergence in plumage ornaments and courtship behavior (Gaston and Jones, 1998; Hunter and Jones, 1999). Least and parakeet auklets lack the aldehyde odorant, suggesting they diverged at a different stage in the phylogeny.

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Chapter 3: Is the citrus-like plumage odorant of crested auklets (*Aethia cristatella*) a defense against lice?[1]

Abstract Plumage odors may function as chemical defenses against ectoparasites in birds. We tested this hypothesis for crested auklets (*Aethia cristatella*), a species of colonial seabird that emits a very strong citrus-like odor from its plumage. This odorant contains known chemical repellents. We evaluated evidence for chemical defense in this species using two approaches. First, we exposed pigeon lice (*Columbicola columbae* and *Campanulotes compar*) to the volatiles emitted by freshly plucked plumage and by whole specimens of recently killed crested auklets. Louse survivorship was compared between these treatments and two controls. Second, we compared louse abundance on crested auklets versus a closely related congener that nests in close association. Louse survivorship did not differ between crested auklet treatments and controls. Comparison of ectoparasite loads showed that crested auklets had significantly higher louse abundance than least auklets (*A. pusilla*), even after controlling for body size. Our results failed to support two expectations for chemical defense. Presence of the crested auklet plumage odorant in our experiments did not reduce louse life span. Presence of the aldehyde odorant in nature did not reduce louse abundance on crested auklets. Hence we conclude

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that the aldehyde odorant is not immediately lethal to lice at natural concentrations in plumage.

Keywords Avian plumage odors, chemical defense, chewing lice, crested auklet, ectoparasite load

Introduction

Strong odors may be indicative of chemical defense in birds (Dumbacher et al. 1992; Dumbacher and Pruett-Jones 1996; Weldon and Rappole 1997), similar to the way in which strong odors are associated with defensive secretions among insects (Eisner 1970). Crested auklets (*Aethia cristatella*) are small (18-20 cm) colonial seabirds from the North Pacific that emit a citrus-like odor (Humphrey 1958). The chemical constituents of the odorant suggest a defensive benefit. Short-chained aldehydes comprise the auklet odorant, and two of the major constituents are potent invertebrate repellents, suggesting that the citrus-like odorant may repel ectoparasites (Douglas et al. 2001). Experiments have shown that synthetic analogues of the crested auklet odorant in very small quantities kill lice and repel ticks (Douglas et al. 2004). This would not be without precedent. Some materials applied topically or sequestered in bird feathers are known to kill parasites. Clayton and Vernon (1993) showed that lime vapor kills lice, and Dumbacher (1999) showed that the toxic feathers of pitohui birds in New Guinea also kill lice. Chemical scents in vertebrates may also be acquired through natural or sexual selection for reasons unrelated to chemical defense (Darwin 1871; Blaustein 1981; Gorman and Trowbridge 1989). This might be true for crested auklets. Conspicuous

sexual and social behaviors suggest that the auklet odorant is important in courtship (Hunter and Jones 1999; Douglas et al. 2001; Hagelin et al. 2003; Jones et al. 2004). Thus, avian plumage odors like the crested auklet odorant might be a form of chemical adornment and not a means of chemical defense. We assessed evidence for chemical defense in crested auklets using two approaches. First, we tested the hypothesis that lice from rock pigeons (*Columba livia*) would not survive exposure to crested auklet plumage as well as to controls. Second, we tested the hypothesis that ectoparasite loads of crested auklets would differ from those of least auklets (*Aethia pusilla*). Our first approach utilized lice from rock pigeons in survival experiments similar to those of Clayton and Vernon (1993) and Dumbacher (1999). The advantage of this experimental design is that pigeon lice can be obtained in large numbers and are known to be killed both by lime vapor (within 9 h, Clayton and Vernon 1993) and pitohui feathers (within 35 h; Dumbacher 1999). We used two species of lice that are host specific parasites of rock pigeons. The two species, *Columbicola columbae* and *Campanulotes compar*, are permanent parasites that are restricted to the body of the host by appendages specialized for locomotion on feathers (Clayton 1991). The two species are "ecological replicates" that complete their entire life cycle on the body of the host, where they feed on feathers and dermal debris (Clayton and Johnson 2003). Transmission among hosts occurs mainly during physical contact between the feathers of different individual birds, such as that between mated individuals or between parents and their offspring in the nest (Marshall 1981).

In our second approach, we determined the ectoparasite loads of individual crested and least auklets caught in the same colony. We performed body washing of individual specimens to assess diversity and abundance of lice. Least auklets live in the same mixed breeding colonies with crested auklets, but do not have a noticeable plumage odor. Least auklets are closely related to crested auklets, and in fact molecular techniques have not been able to resolve the phylogeny of the four *Aethia* auklets (Friesen et al. 1996). At our research site, crested and least auklets socialize on some of the same landing rocks, and nest near each other in rock crevices.

Methods

Survival experiments

We tested the hypothesis that the crested auklet's plumage odor kills ectoparasitic lice (Phthiraptera:Insecta). We conducted two experiments on St. Lawrence Island, Alaska, during August 2001. In experiment I, we filled petri dishes with freshly plucked feathers from the nape and upper back of freshly killed crested auklets. In experiment II, we placed whole specimens in beakers. Each experiment had three treatments—crested auklet, least auklet, and rock pigeon. Experiment I used three replicates for each treatment. Experiment II used three replicates for crested auklets and least auklets but only two replicates for rock pigeons. Crested auklet treatments continued to emit the characteristic citrus-like odor throughout the trials in both experiments, but the other treatments did not emit a noticeable odor. Experiment I had the advantage of concentrating the feathers and their associated volatiles within a small volume.

Experiment II had the advantage of using whole specimens that probably retained the aldehyde odorant for a longer period and emitted more of the chemical. However, in experiment II the volatile chemicals would have diffused in a larger air volume. In both experiments the lice were placed on a piece of porous filter paper suspended just above the feathers, preventing direct contact by lice, similar to the design used by Clayton and Vernon (1993). This approach allowed us to keep track of individual lice and controlled for any differences in survival owing to differences in the substrate itself, rather than odor, *per se*.

Survival experiments: petri dishes

Experiment I Contour feathers were plucked from the nape and upper back of a recently killed bird (within 8 h of death). Sufficient feathers were plucked to fill the base of a petri dish. The feathers were covered in the base of the dish with a piece of porous filter paper. Six *Columbicola columbae* and six *Campanulotes compar* were placed on top of filter paper covering the feathers, and the petri dish was covered with a lid. In two replicates there was one fewer louse due to shortage. The two species of lice were pooled as a single category. Survival of the lice was determined after a period of 30 h by examining each louse closely under a dissection microscope. A few lice managed to escape from the dishes during the 30-h trial. The rate of escape was independent of treatment, so those lice that escaped were reported in our results but excluded from the statistical analysis of survival.

Survival experiments: beakers

Experiment II This experiment also had three treatments with three beakers per treatment for crested and least auklets, and two beakers for the rock pigeon treatment (because we had only two pigeons). A recently killed bird of the relevant species was placed in each beaker at the start of the experiment. Ten lice of each species were placed on top of filter paper suspended in the mouth of the beaker, which was then sealed with aluminum foil. In one replicate there was one extra louse. Again, the two species of lice were pooled as a single category. Survival of the lice was determined after a period of 30 h using the same criteria as for the petri dish experiment.

Comparison of natural louse loads

We compared the relative louse abundances on 21 crested and 25 least auklets sacrificed for other studies. These specimens were obtained from the same nesting area in the Myauk colony on St. Lawrence Island, Alaska, on 4–5 August 2001. Specimens were immediately placed inside individual paper bags and sealed in ziploc plastic bags. The specimens were shipped frozen to the Clayton laboratory at the University of Utah where louse loads were quantified using the body washing method of Clayton and Drown (2001). Lice were tabulated for each specimen and identified to species (*Quadraceps aethereus*, *Saemundssonia wumisuzume*, *S. boschi*, *Austromenopon nigropleurum*). The same species of *Quadraceps* and *Austromenopon* occur on crested and least auklets. *S. boschi* (a new species of avian louse) was found only on least auklets, and *S. wumisuzume* occurred on crested auklets but not least auklets (Price et al. 2003). Data on

ectoparasite loads were adjusted for body mass because crested auklets (mass=252 g, SE=3.6, n=21) averaged three times larger than least auklets (mass=84 g, SE=1.2, n=17). The number of lice on least auklets was multiplied by a numerical coefficient (2.17) derived from Clayton and Walther (2001, Fig. 2b), who demonstrated a predictable relationship between avian ectoparasite load and body size of the host in a study of 52 species of Peruvian birds. The corrected data for least auklets were compared to ectoparasite loads of crested auklets with a Mann-Whitney U test.

Results

Survival experiments: petri dishes

Fewer lice escaped from petri dishes with crested auklet plumage (17.6%) than those with least auklet (22.2%) and rock pigeon plumage (33.3%), but this difference was not significant (χ^2=2.5, df=2, P=0.29, Table 3.1). Louse survival did not differ between treatments either (Table 3.1): 42.9% of lice over crested auklet feathers survived for 30 h, compared to 39.3% of lice over least auklet feathers, and 41.7% of lice over the (control) rock pigeon feathers (χ^2=0.08, df=2, P=0.96).

Survival experiments: beakers

More lice escaped from the filter paper in the least auklet treatment (30%) than those lice in the crested auklet (9.8%) or rock pigeon (10%) treatments (χ^2=10.6, df=2, P=0.005, Table 3.1). It was not possible to positively determine the fate of escaped lice, and we do not know why more lice escaped from the least auklet treatment. The observation

demonstrates that lice were capable of climbing and escaping from the filter paper. Survival of the remaining lice among the three treatments in this experiment was even more similar than in the previous experiment: 47.3% of lice over crested auklets survived for 30 h, compared to 47.6% of lice over least auklets, and 44.4% of lice over rock pigeons. Again, the differences were not statistically significant (χ^2=0.09, *df*=2, *P*=0.95). Survival experiments were ended after 30 h because there were no apparent trends in mortality, some lice had escaped, and the potency of the auklet feathers had likely diminished over time.

Table 3.1 Results of louse survival experiments on feathers and specimens of crested auklets (*Aethia cristatella*), least auklets (*A. pusilla*) and rock pigeons (*Columba livia*)

Treatment	Stayed	Escaped	Totals		Alive	Dead	Totals	
Exp. 1: Petri Dishes (feathers)					Survival results lice that stayed			
Crested auklet	28	6 (17.6%)	34	$\chi^2 = 2.5$	12 (42.9%)	16 (57.1%)	28	$\chi^2 = 0.08$
Least auklet	28	8 (22.2%)	36	$df = 2$	11 (39.3%)	17 (60.7%)	28	$df = 2$
Rock pigeon	24	12 (33.3%)	36	$P = 0.29$	10 (41.7%)	14 (58.3%)	24	$P = 0.96$
Exp. 2: Beakers (specimens)					Survival results lice that stayed			
Crested auklet	55	6 (9.8%)	61	$\chi^2 = 10.6$	26 (47.3%)	29 (52.7%)	55	$\chi^2 = 0.09$
Least auklet	42	18 (30.0%)	60	$df = 2$	20 (47.6%)	22 (52.4%)	42	$df = 2$
Rock pigeon	36	4 (10.0%)	40	$P = 0.005$	16 (44.4%)	20 (55.6%)	36	$P = 0.95$

Comparison of ectoparasite loads

Crested auklets had higher louse loads than least auklets (Mann-Whitney: $P<0.001$, two-tailed). Two species of louse were more abundant on crested auklets than least auklets—*Q. aethereus* (Mann-Whitney $P<0.001$, two-tailed), *Saemundssonia* sp. (M-W, $P<0.001$, two-tailed). However, abundances of *A. nigropleurum* did not differ between crested and least auklets (M-W $P=1.0$, two-tailed). Even after adjusting for differences in host body size, crested auklets still had significantly more lice than least auklets (M-W $0.009<P<0.01$, two-tailed). In this comparison, the difference was principally due to a higher abundance of *Q. aethereus* (M-W $0.005<P<0.006$, two-tailed) on crested auklets. The abundances of other louse species did not differ between crested and least auklets (M-W *Saemundssonia* sp., $0.09<P<0.1$, *A. nigropleurum* $0.2<P<0.3$, two-tailed).

Discussion

There was no support for the predicted differences in 30-h louse survival or comparative louse abundances. Exposure of lice to volatiles from crested auklet plumage did not reduce the life span of pigeon lice. Survivorship of lice was similar regardless of whether the lice were placed with plumage of least auklets, crested auklets, or rock pigeons. Lice also were more abundant on crested auklets than least auklets, even after adjusting for host body size. This demonstrates that the presence of the aldehyde odorant in nature does not reduce louse abundance relative to a congeneric dwelling in close association at the same colony. Observations collected during the experiment suggest that lice may not have been repelled by the crested auklet treatment. We noted that fewer lice escaped from

the crested auklet treatments than the controls. This might have happened because subtle differences in the placement of the filter paper allowed more lice to escape from some beakers and dishes. Alternatively, fewer escapes might be indicative of impairment of lice by the crested auklet odorant, thus reducing their mobility.

Our results suggest that the crested auklet odorant does not function as a chemical defense. However, there are caveats. Abundances of lice were higher on crested auklets than least auklets. This might be attributable to differences in sociality. Crested auklets are highly gregarious, more so than least auklets (Gaston and Jones 1998). This brings crested auklets into close contact with each other on a frequent basis, and this contact affords increased opportunities for louse transmission. Furthermore, our experiments may not reproduce the same effects as live birds. First, lice in our treatments did not come in contact with plumage. Second, the aldehydes are ephemeral in plumage due to their volatile and reactive qualities. Trials with synthetic analogues of the crested auklet odorant show that the efficacy of the odorant as a repellent begins to wane 1 h after application (Douglas et al. 2004). This is similar to what has been shown for commercial repellents such as DEET (Dautel et al. 1999). Interpretation becomes further complicated when we consider that the crested auklet odorant may require a longer period to act than the duration over which the experimental data were collected. Pitohui feathers of lower toxicity required longer periods to inflict mortality (>100 h for some louse species; Dumbacher 1999). Synthetic analogues of the crested auklet odorant are strongly repellent to ticks, and exposure to very small quantities kills lice instantly (Douglas et al. 2004). However, these effects are dose dependent, and the transience of the odorant may

have resulted in reduced dose over the period of our trials. Constituents of the crested auklet odorant are lethal to auklet lice (Douglas et al. 2004). However, our results suggest that the crested auklet odorant is not immediately lethal to lice at naturally occurring concentrations. Instead, the odorant may have sublethal effects such as repellence, impairment, or delayed development.

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Chapter 4: Chemical odorant of colonial seabird repels mosquitoes[1]

ABSTRACT The crested auklet (*Aethia cristatella*) emits a class of aldehyde chemicals shown to be potent invertebrate repellents when used by Heteropteran insects against their predators. Our aim was to determine the efficacy of these aldehydes against mosquitoes in the laboratory. Synthetic analogues of the auklet odorant were strongly repellent to mosquitoes (*Ae. aegypti*) in controlled laboratory trials. Furthermore, the efficacy was similar to previous reports for commercial mosquito repellents. These results, in combination with a previously published study, show that constituents of the aldehyde odorant are broad spectrum in efficacy against ectoparasitic arthropods of birds. Our report is the first empirical evidence for an endogenous mosquito repellent in birds.

KEY WORDS mosquito repellent, chemical defense, aldehydes, ectoparasites, crested auklet

Introduction

Avian chemical defense has been discussed in the context of ectoparasites (Simmons 1966, Ehrlich et al. 1986, Clayton and Vernon 1993, Dumbacher 1999), but heretofore no published experiments have specifically addressed mosquitoes. We

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suggest that birds may use endogenous or exogenous chemicals to repel mosquitoes and other ectoparasites in a manner analogous to human and nonhuman primates (Berenbaum 1995, Valderramma et al. 2000, Weldon et al. 2003). Synthetic and botanically-based repellents are widely employed by humans against mosquitoes and other ectoparasites. Some of these products are broadly effective against ectoparasites as a group. The commercial market for such products apparently exploits human behaviors that have ancient origins (Smith and Secoy 1975, Smith and Secoy 1976, Secoy and Smith 1983, Hillocks 1998). Nonhuman primates are reported to self-anoint with naturally occurring repellents effective against mosquitoes (Valderramma et al. 2000, Weldon et al. 2003). Similar behaviors have been widely reported in birds where some species apply naturally occurring chemical repellents to their plumage and nests (e.g., crushed ants, citrus peel, marigold flowers, volatile green plants) as possible defenses against lice, mites, bacteria, and fungi (Ehrlich et al. 1986, Clark and Mason 1985, Clark and Mason 1988, Clayton and Vernon 1993, Moyer and Clayton 2003). Observations of endogenous chemical defense in birds are exceedingly rare; the pitohuis and *Ifrita kowaldi* of New Guinea are the best known examples (Dumbacher et al. 1992, 2000; Dumbacher 1999). The pitohuis emit a sour odor, possibly as an olfactory signal of poison, analogous to their aposematic coloration (Dumbacher et al. 1992). Other avian species also emit odors, and these odors may be chemical defenses or indicative of chemical defenses (Weldon and Rappole 1997).

Crested auklets (*Aethia cristatella*) are small planktivorous seabirds of the North Pacific that emit a noticeable chemical odorant during the breeding season (May-August,

depending upon latitude). The dominant constituents of this odorant are octanal and hexanal (Douglas et al. 2001, Douglas et al. 2004). These compounds are also found in the metasternal gland emissions of heteropteran insects (Blum 1981). Heteropteran secretions containing octanal and hexanal are potent invertebrate repellents (Aldrich 1988), and the compounds have similar effects upon ectoparasites. For example, synthetic analogues of the crested auklet odorant are strongly repellent to ticks (*Amblyomma americanum, Ixodes uriae*), and a single microliter applied to a feather kills lice (genera *Austromenopon* and *Quadraceps*) within seconds (Douglas et al. 2004). However, the life spans of avian lice suspended above crested auklet feathers were not reduced relative to controls (Douglas et al. 2005). Nevertheless, brief exposure of lice to shallow incisions made in the skin of a freshly killed crested auklet instantly killed or paralyzed lice, suggesting that the auklet odorant may at least have an inhibitory effect on lice at natural concentrations in plumage (Douglas 2005).

In the research reported here we tested the efficacy of the crested auklet's odorant against mosquitoes. It was noted that mosquitoes attempt to parasitize auklets at St. Lawrence I., Alaska (H. Douglas, pers. obs.). Some of the auklet colonies on this island occur in large rock talus fields, surrounded by moist tundra, and the close proximity apparently provides a suitable habitat for mosquitoes. We tested the hypothesis that constituents of the crested auklet's odorant repel mosquitoes. We herein show that a synthetic analogue of the odorant was strongly repellent to mosquitoes in standardized laboratory trials.

Materials and Methods

Mosquito repellency trials with laboratory reared and maintained *Aedes aegypti* (Linnaeus) (Vero Beach strain) female mosquitoes were conducted from 24-28 February 2002 at the Florida Medical Entomology Laboratory, Vero Beach, Florida. This species was selected as the test mosquito because it is an extremely aggressive biter, is active during the day, and readily blood feeds on a variety of hosts, including humans, in the laboratory. This makes *Ae. aegypti* an ideal candidate for repellency trials. Mosquitoes were exposed 10 at a time to synthetic constituents of the crested auklet's aldehyde odorant in 30 x 23 x 22 cm test cages maintained under natural light, temperature, and humidity. Test mosquitoes were discarded after each experimental trial and replaced with 10 new females that had not been previously exposed to test solutions.

In each trial, a small amount of substance (0.6-0.8 ml) was added to a circular filter paper 9 cm in diameter (Whatman Qualitative 2). The chemicals were allowed to volatize until the paper was dry (5-8 min). The filter paper was then laid on top of two untreated filter papers and attached to the bare skin on the back of H. Douglas' hand with a rubber band. The rubber band was wrapped around a closed fist so that the filter paper completely covered the back of the hand. We used the intensity of mosquito host-seeking behavior and mosquito landings on the treated filter paper as an index of repellency. This was similar to the methods described by Fradin (1998) where repellent chemicals caused female mosquitoes to suspend their search for blood. To quantify repellency we counted the number of times mosquitoes alighted on a hand treated with auklet odorant diluted in ethanol compared to a hand treated with pure ethanol. Counts were made within

consecutive 15 second periods during a 5 min observation period. For each trial the hand was gently placed into a mosquito test cage used for repellent testing in the laboratory (Fradin and Day 2002). During repellency experiments the treated hand was rested on the floor of the cage so that the filter paper covering the back of the hand was the main exposed target. After alighting mosquitoes were lightly shaken off and were not permitted to blood feed. Every 15 sec the hand was rolled over to search for and dislodge mosquitoes that might have landed on the underside. Female mosquitoes were exposed to an untreated hand prior to daily experiments to verify their willingness to blood feed.

Four repellency experiments were conducted with synthetic constituents of the crested auklet's odorant. In Experiment 1, individual odor constituents (Hexanal, Octanal, Z-4 Decenal, Hexanoic Acid) were diluted to approximately 90% concentration (9 parts active ingredient:1 part ethanol), and the intensities of mosquito attacks were compared to an ethanol control (100% purity). This was approximately the same or slightly lower purity than odor production in crested auklets (e.g. the octanal that crested auklets emit in nature is higher than 92 % purity; Douglas et al. 2001). One experimental trial was conducted for each treatment. Experiment 2 exposed mosquitoes to a synthetic blend of the crested auklet odorant according to measurements of relative abundances (40% Octanal, 21% Hexanal, 8% Z-4 Decenal, 8% Hexanoic Acid, 3.5% Decanal, 3.5% Octanoic Acid; Douglas et al. 2001, 2004). This was performed at three concentrations (1, 10,100% with 3, 4, 3 replicates, respectively). The results were graphed in a dose-response curve. Experiment 3 compared repellency of a single ingredient, octanal, versus the combined blend at 2.5% concentration (with 4 replicates), to test for synergistic

properties of the blend. Experiment 4 compared the repellency of a carboxylic acid (hexanoic acid) versus the corresponding aldehyde (hexanal) at 2.0% concentration (with 3 replicates). The results from Experiment 1 were analyzed with a One-Way ANOVA and multiple comparisons using a Bonferroni correction (ProStat v. 3). Chi-square analyses were applied to the results of Experiments 2-4 (ProStat v. 3).

We used the following synthetic chemicals in these experiments: Hexanal 96% (C.A.S.: 66-25-1), Octanal 99% (C.A.S.:124-13-0), Decanal 95% (C.A.S.:112-31-2), Hexanoic Acid 99+% (C.A.S.:142-62-1), Octanoic Acid 99% (C.A.S.:124-07-2) by ACROS Organics (Pittsburgh, PA) and Z-4 Decenal 95% (C.A.S.: 21662-09-9) by Lancaster Synthesis (Windham, NH). These constituents were presented individually and combined in a mixture at different dose levels. Ethanol 100% (C.A.S.:64-17-5) was used as a solvent for diluting the odorant constituents. Ethanol also served as the control treatment. It is important to note that Octanal is often confused with various chemical forms of eight carbon alcohols, such as Octenol. Octanal is an eight-carbon aldehyde with repellent properties, while Octenol is an eight-carbon alcohol which is a strong attractant for many biting arthropod species (Kline 1994).

Results

In Experiment 1, auklet odorant constituents were more repellent to mosquitoes than was the ethanol control (Table 4.1). Mosquitoes avoided the filter paper in experimental treatments, and those few that landed alighted on exposed skin. Mosquitoes did not exhibit the same avoidance to identical filter papers treated with ethanol. Instead,

many more mosquitoes landed on the control treatment (ANOVA $F_{0.05(4,95)} = 108.72$, $p<0.0001$). All of the experimental treatments were more repellent than the ethanol control ($p<0.001$), but none were significantly different from each other (p ranging from 0.43 to 0.91).

Table 4.1. Mean mosquito landings per 15-second interval (20 intervals) for crested auklet odorant constituents versus an ethanol control.

Chemical (9 pts:1 pt ethanol)	**Landings**	**Std. Error**	***P*** **(vs. control)**
Hexanal	0.20	0.09	<0.001
Hexanoic Acid	0.35	0.17	<0.001
Octanal	0.15	0.08	<0.001
Z-4 Decenal	0.00	0.00	<0.001
Ethanol Control	7.45	0.67	

The intensity of mosquito attacks increased in Experiment 2 when the dose level of the auklet odorant was reduced from 10% to 1% concentration (Fig. 4.1; $\chi^2 = 10.0$, df = 2, $p = 0.006$), but there was no significant difference between 10% and 100% concentrations ($\chi^2 = 0.07$, df=2, $p = 0.97$).

There were no significant differences for treatments in Experiments 3 and 4. Octanal was not more repellent than the aldehyde mixture ($\chi^2 = 0.94$, df = 3, p = 0.82) in Experiment 3 (Table 4.2). In Experiment 4, the mosquito landing response to 2.0% hexanal and hexanoic acid was equivalent (0.15-0.75 vs. 0.15-0.70, respectively; $\chi^2 = 0.08$, df=2, p = 0.84).

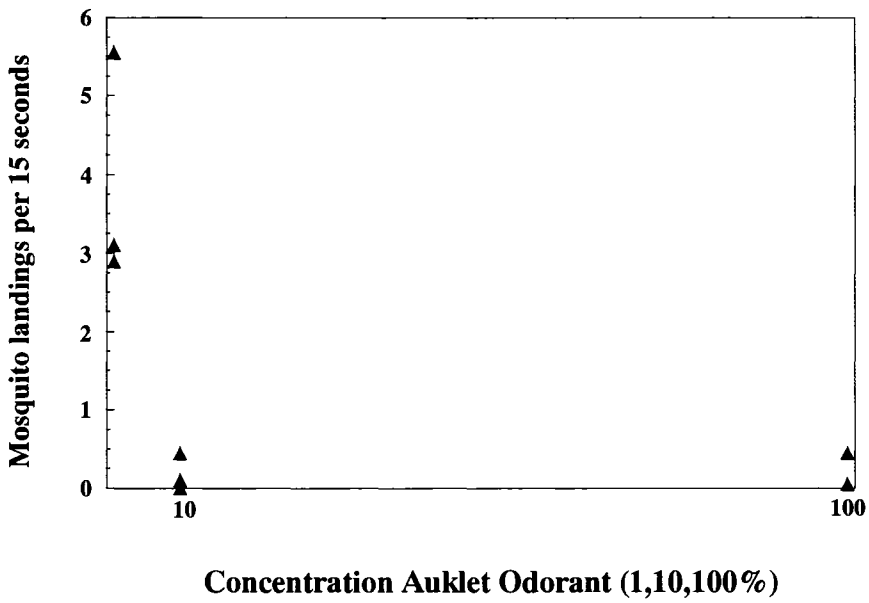

Figure 4.1. Mosquitoes exhibited a dose-dependent response to synthetic analogues of the crested auklet odorant. Response is recorded as the mean number of mosquito landings per 15 second intervals for three doses (1, 10,100% with 3, 4, 3 replicates, respectively).

Ethanol control experiments conducted at the beginning and end of these experiments confirmed that mosquito attack response was equivalent through the course of this research $t\ (19)_{1\text{-tailed}} = 1.20$, $p < 0.122$. Mosquitoes attacked the ethanol control more strongly than all experimental treatments, including the 1% crested auklet aldehyde blend ($x = 4.22 \pm 0.38$; $t\ (78)_{2\text{-tailed}} = 5.03$, $p < 0.001$; Tables 4.1 and 4.2).

Table 4.2. Comparison of mosquito response to the crested auklet odorant versus octanal, each at 2.5 percent concentration in ethanol.

	2.5% Octanal		**2.5% Auklet Odorant**	
	Landings/15 s.	S.E.	Landings/15 s.	S.E.
Trial 1	0.95	0.38	2.8	0.37
Trial 2	0.45	0.18	0.55	0.17
Trial 3	0.1	0.1	0.05	0.05
Trial 4	0.15	0.08	0.15	0.08

Discussion

The aldehyde odorant of the crested auklet has characteristics similar to those reported for commercial mosquito repellents in comparable laboratory tests (Fradin and Day 2002). Test mosquitoes ceased to fly after brief exposure to a synthetic analogue of the crested auklet odorant and soon became inactive on the sides of the cage. This

behavior was also observed in test mosquitoes in a similar research design used to evaluate commercial repellents containing N, N-Diethyl-meta-toluamide (DEET) as the active ingredient (Fradin and Day 2002). Wild crested auklets emit nearly pure aldehydes in nature (Douglas et al. 2001), and the odor of one bird smells at least as strong as the low doses used in Experiments 2-4 (H. Douglas, pers. obs.). In previous bioassay trials we noted that low concentrations of the auklet odorants, like commercial repellents (e.g., DEET), were strongly repellent to ticks (Douglas et al. 2004), but efficacy of the auklet odorant began to wane one hour after application. This transience of repellent efficacy has also been observed in experiments using DEET with ticks (1% active ingredient, *Ixodes ricinus* Linne 1758 (Dautel et al. 1999) and mosquitoes (5-7% active ingredient, *Ae. aegypti*, Fradin and Day 2002).

Evolution of the crested auklet odorant may be linked to arthropod avoidance. Ectoparasite avoidance in nature directly benefits individual fitness of vertebrates because ectoparasites can transmit diseases and can also impose a blood-feeding burden on hosts (Cotgreave and Clayton 1994). Even near marine environments, ectoparasite infestations of seabird colonies have been associated with disease, mortality, breeding failures, mass abandonment of colonies, reduced reproductive success, and reduced growth rates in chicks (Feare 1976, Anderson and Fortner 1988, Duffy 1993, Ramos et al. 2001, Moyer and Clayton 2003). At the very least the chemical repellency of the auklet aldehyde blend may interfere with the host/parasite interface thereby reducing the frequency and annoyance of blood feeding by ectoparasites and the resulting probability of disease transmission. Chemical constituents of the crested auklet's odorant appear to

have broad spectrum efficacy against ectoparasitic arthropods. Parasites vary in their geographical and temporal distribution and effects (Clayton and Moore 1997, Moore 2002), so a chemical defense that is effective against more than one parasite may be more successful in evolutionary terms.

The crested auklet's odorant is similar to naturally occurring substances that birds apply to their plumage and nests. Similar defensive chemicals are found in arthropods and plants (Eisner 1970), and terrestrial birds use both arthropods and plants as natural prophylactics to anoint their plumage. Many bird species crush and rub ants through their plumage, thereby obtaining corrosive chemicals from the ant secretions (Simmons 1966, Ehrlich et al. 1986, Clayton and Vernon 1993). Birds are also known to rub plant materials through their plumage, including citrus fruit, apparently as a defense against lice (Clayton and Vernon 1993). Constituents of the crested auklet odorant are corrosive chemicals. Some of these same chemicals are present in the outer rind of citrus fruit (Morton 1987) and the metasternal gland emissions of Heteropteran insects (Aldrich 1988). In both cases these chemicals are thought to have defensive functions (Morton 1987, Aldrich 1988).

Birds exhibit behavioral defenses in response to mosquito biting pressure (Edman et al. 1974, Scott and Edman 1991); however endogenous avian chemical defenses against mosquitoes have not been previously demonstrated. Evidence from primates suggests that some self anointment behaviors of birds might be effective against mosquitoes. Capuchin monkeys (*Cebus* spp.) anoint themselves with millipedes, apparently for benefits of mosquito repellency (Valderramma et al. 2000, Weldon et al.

2003). A similar anointing behavior with millipedes has been noted in birds (Clunie 1976). Reports of these types of behavior, termed "anting," are particularly prevalent during warm weather and high humidity (Ehrlich et al. 1986), conditions that support blood-feeding by mosquitoes. We suggest these behaviors could be explored as behavioral sequestration of exogenous chemical defenses, and such explorations may yield new natural products with repellent properties.

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Chapter 5: Measuring chemical emissions in crested auklets (*Aethia cristatella*)[1]

Abstract— The first quantitative estimates of emission rates for chemical signals in a bird - the crested auklet (*Aethia cristatella*) are presented herein. Volatile emissions from live birds were captured in a purified air stream onto polymer traps. Traps were eluted with methanol and analyzed with GC-MS. The volatile collection chamber was field calibrated with an in-line bubbler and synthetic octanal, the dominant constituent of the crested auklet's citrus-like odor. The result is an index of volatile chemical emissions within a small population of wild crested auklets at Big Koniuji I., AK. The average emission rate for octanal was 5.7 μl/50 min. Males and females did not differ in their mean emission rates and ranges. There was a sevenfold difference between males with the minimum and maximum emission rates. Prevalence of tick infection (2.1%) was low despite high abundance of ticks in the colony. The crested auklet with the lowest chemical emission rate had 14 ticks attached to the face, whereas nearly all other crested auklets had no ticks.

Key Words – Crested auklet, chemical emissions, chemical signal, octanal, ectoparasites, repellents, chemical defense.

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INTRODUCTION

Chemical signals in vertebrates can provide information about the parasite resistance of prospective mates, and such signals may be amenable to sexual selection (Penn and Potts 1998). Many species of birds emit odors (Weldon and Rappole, 1997), but the functional significance of avian odors has received relatively little attention. Weldon and Rappole (1997) suggested that avian odors may be indicative of chemical defense or unpalatability. In birds, few endogenous chemical defenses have been documented, but exogenous defenses, applied to plumage and nests, may be widespread (Simmons 1966; Clark and Mason, 1985, 1988; Ehrlich et al., 1986; Clayton and Vernon, 1993; Gwinner et al., 2000; Lafuma et al., 2001; Parkes et al., 2003; Weldon, 2004). The best known examples of endogenous chemical defense among birds are the Pitohuis and *Ifrita kowaldi* of New Guinea (Dumbacher et al., 1992, 2000). These species apparently sequester batrachotoxins, a potent class of neurotoxins, from dietary sources, particularly Choresine beetles (Family Melyridae; Dumbacher et al., 2005). The concentration of batrachotoxins can be sufficient in skin and plumage to act as a deterrent against parasites and predators (Dumbacher et al., 1992, 2000; Dumbacher, 1999). However, chemical characteristics of these neurotoxins vary widely within and among species, consistent with evidence that the substances are acquired from an environmental source (Dumbacher et al., 2000). Two species of pitohuis ("hooded" *P. dichrous* and "variable" *P. kirhocephalus*) emit a sour odor that is hypothesized to serve as an olfactory warning of the birds' poisonous characteristics (Dumbacher et al., 1992). The chemical odors of pitohuis have not yet been characterized qualitatively or quantitatively.

Crested auklets (*Aethia cristatella*) produce a citrus-like odorant dominated by even-numbered aldehydes (6-12 carbons) that may function as an ectoparasite repellent and signal of mate quality (Douglas et al., 2001, 2004). Two of the primary constituents are known to be potent invertebrate repellents, and synthetic analogues of the crested auklet odorant repel, paralyze and kill ectoparasites in a dose-dependent fashion (Douglas et al., 2001, 2004, 2005b). Efficacy of synthetic analogues of the crested auklet odorant is comparable to that of commercial arthropod repellents (Douglas et al., 2005b). Brief exposure of auklet lice to *in situ* concentrations of the crested auklet odorant in tissues caused paralysis and mortality; however, suspension of pigeon lice above crested auklet feathers had no effect on survivorship compared to controls (Douglas et al., 2005a).

This report presents an accurate method for comparison of odor production in live crested auklets that can also be applied to other vertebrates. In this method, wild birds were captured and confined in a purified and regulated air stream while chemical emissions were captured onto polymer traps. Portable industrial instruments designed for detection of volatile organics are not sufficiently specific or accurate to provide comparative measurements. In crested auklets, chemical concentrations vary considerably within an individual bird's plumage (H. Douglas, unpubl. data; see Chapter 8). A large sample of feathers is required to obtain an accurate average measurement for each body region, but this would compromise the thermal insulation of the birds and cause mortality. Therefore, quantitative comparisons of individual chemical potency with feather extracts will require that birds be sacrificed. Also, crested auklet colonies are located in windy environments. The sealed chamber eliminates the problem of

variability in detections that would be caused by wind blowing over the surface of plumage.

METHODS AND MATERIALS

Field Methods. Research on the chemical odor of crested auklets was conducted at a colony on Big Koniuji I., AK, from June 4 - July 16, 2002. In terms of phenology, this time period corresponded with the onset of egg-laying to early chick rearing. The colony is situated in an ancient glacial cirque at 243 m elevation on a mountain overlooking Yukon Harbor. The crested auklet nests in rock talus, high on the steep slopes of this cirque. It is the only seabird species that nests in the cirque. Breeding adults synchronized visitations each morning during the incubation period, gathering in a large flock in Yukon Harbor prior to visiting the colony. Crested auklets arrived on the colony surface in a large flock and were captured in noose carpets strung over landing rocks. Each bird was measured, banded with a USFWS metal band, color bands, sampled, sexed by bill characteristics (according to Jones, 1993a), and inspected visually for ectoparasites.

Each bird (n=57) was then placed in a glass reaction kettle, and volatile emissions were collected in a purified air stream. The flow rate of 1.0 L/min delivered oxygen at a rate of 0.2 L/min (dry atmospheric air=20.95% oxygen, Schmidt-Nielsen 1997), which was more than sufficient to supply the oxygen consumption needs of a 300 gram non-passerine bird (0.25 L/hr at rest; Lasiewski and Dawson, 1967). Air temperature during volatile collections was 6-10° C. Duration of sampling was measured with a stopwatch.

Volatile emissions were collected for 50 min on all except six birds. The period of volatile collection for those six was reduced to 30 min in order to expedite research. Gas chromatography peak areas for octanal were adjusted to account for the difference in collection times. The adjusted values for those six individuals lay within the range of other values in the sample. After GC/MS analysis, peak values for all samples were normalized to a 50 min collection time (CT) with the following equation: 50 min/CT * peak area = adjusted peak area. Collection traps were eluted with 2.0 ml of methanol, and the elution was collected in borosilicate glass vials (3/8 oz., Fisher Scientific), sealed with Teflon-lined caps and a vapor seal (DuraSeal stretch film). The chambers were scrubbed with baking soda, rinsed with freshwater, and dried with a clean cotton towel between each sampling.

Design of volatile collection system. The design of this system benefited from the study of similar methods used to study plant volatiles (e.g., Turlings et al., 1991). The volatile collection chamber (Fig. 5.1) consisted of a 4000 ml Pyrex reaction kettle (Corning #6947-4L) and kettle lid (VWR 36393-051) clamped securely with a kettle clamp (VWR Cat. No. 36393-051). All tubing and fittings were glass or Teflon. The incoming air stream was filtered through a glass dispersion tube (Lab Glass UA-71801-11950, extra coarse porosity 170-220) and then an in-line charcoal filter (Whatman Carbon Cap). This purified air stream entered the reaction chamber through a 24/40 glass elbow (LabGlass LG-1980-100). The outgoing air stream was split, exiting through two 24/40 glass elbows into two glass collection traps. The collection traps were custom manufactured by Lab Glass (Vineland, NJ) and adapted from a design described in

Turlings et al. (1991). Each collection trap was 6 cm in length and 9 mm in outer diameter (6 mm I.D.). The tube was fitted with a 325-mesh stainless steel frit sealed across the diameter of the tube, 20 mm from the downstream end. Glass collection traps were packed with Super Q (80-100 mesh; Alltech, Deerfield, Illinois) or Tenax (35/60 mesh; Alltech). These traps were conditioned under vacuum in a Bullet Dryer at 225° C for 14 hours prior to shipping to the field site. The polymer (50.0 mg) was placed on top of the frit and held in place with a small plug of glass wool. Air exiting from these collection tubes was passed through Gilmont flow meters hooked up to a battery operated vacuum source (Cole Parmer Model# 7530-25). Flow rate was regulated at 500 ml/min through each collection trap. Flow meters were factory calibrated prior to the field season, and calibration of the flow meters was checked again after fieldwork. This calibration was performed with Sierra 820 Mass Flowmeter, and standard error was ± 0.41 ml/min. The volatile collection system was calibrated in the field by passing 3.0 ml of synthetic octanal through a bubbler, placed in line (Ace Midget Bubbler with 145-175 micron filter, Ace Glass Inc., Vineland, NJ). This calibration was run exactly as performed with crested auklets, with the volatile collection system operating for 50 min. The calibration was performed once at the end of the field season to avoid contamination of equipment and samples with standards. Blank controls were also run once at the end of the season without a bird in the chamber.

Chemical analysis. Gas chromatography-mass spectrometry was carried out in the SIM mode (selective ion monitoring) using a HP5890 Series II Gas Chromatograph equipped with a 20 m x 0.25 mm, 5% phenyl siloxane column (Alltech), and a HP5972

Series Mass Selective Detector. The injector and detector temperatures were held at 250° C throughout, and the column flow was 1.0 ml/min. The instrument was programmed from 60 to 250° C in two stages. The first level increased at a rate of 4°C/min to a final temperature of 120° C and remained at that temperature for 4 min. The second level increased at a rate of 8° C/min to a final temperature of 250° C and remained at that temperature for 2 min. Mass spectra were obtained with an HP 5972 Series Mass Selective Detector. Octanal was selected as an index of chemical potency because it is consistently the most abundant constituent in the crested auklet odorant (40%, Douglas et al., 2001), and it is also strongly repellent to ticks (Douglas et al., 2004). Retention time and ion abundances were obtained in EI mode from standard (99% Octanal, ACROS Organics, C.A.S. 124-13-0), and results were consistent across five replicates at different concentrations. The most abundant ions were chosen for monitoring, and dwell times (milliseconds) were set for each ion according to its relative abundance (ion/dwell time: 43.0/100; 41.0/80; 44.0/70; 57.0/30; 84.1/30). Subsequent analyses of standards in SIM mode showed that these parameters consistently discriminated octanal from background and obtained well-defined peaks.

Quality control was assured by the inclusion of blanks, duplicates, internal standards, augmented standards, and calibration standards run at intervals in sequences at the frequency of 5-10% of total samples. Standards were made by serial dilutions in methanol (ACROS Organics HPLC grade). Undecenal (97% Undecyclenic Aldehyde, ACROS Organics, C.A.S. 112-45-8) was used as an internal standard, and this internal standard was added to all samples. Blanks with the internal standard were also included

in the sequence. Precise quantities of standards were measured with an Eppendorf Pipette (Model 4710). Accuracy and precision of the pipette were determined by replicate weighing of 10 µl samples of distilled water with a Mettler AE163 analytical balance ($x = 9.94 \times 10^{-3}$ g; S.E.= $\pm 8.38 \times 10^{-5}$; S.D. = 3.25×10^{-4}).

Instrument variability of the GC/MS was addressed by calibrating response factors of the target analyte and the internal standard. A series of calibrations was conducted at four concentrations of octanal and undecenal (3.8×10^{-3} µl/ml, 4.8×10^{-3} µl/ml, 6.0×10^{-3} µl/ml, 9.0×10^{-3} µl/ml) to obtain the regression of relative response ($R^2 = 0.96$, $p < 0.0001$, $n = 22$). The following regression equation was obtained:

Octanal Peak Area = 7.58 * (Undecenal Peak Area) + 14135

For each sample, I calculated the difference between the obtained and expected GC peak areas of the internal standard. This difference in undecenal peak area was applied in the regression equation above, and the value was added to the obtained value for octanal.

Duplicate samples were collected for all birds. However, the Big Koniuji colony is located in a rugged spot, and some samples were lost due to damage to the sample vials. Some samples were expended in the testing and calibration of analytical methods. I analyzed 103 samples from 57 individuals -- 46 individuals with duplicate split samples and 11 individuals with single split samples. Results obtained for Tenax and Super-Q volatile traps in the 46 duplicate split samples were similar. Standard error of splits, expressed as a percentage of peak area, was 8 percent. In the case of duplicate split samples, average octanal peak area was used as a relative index for chemical potency. In the cases where only single splits were available, the obtained value for octanal peak area

(± 8%) was used as the relative index. Quantitative measurements, obtained from field calibration with synthetic octanal and bubbler, were used to calculate emission rates for the birds.

Evaluating ectoparasite abundance. A visual inspection method, similar to that described in Clayton and Walther (1997), was used as a relative measure of ectoparasite prevalence and abundance on crested auklets measured for chemical emission. A subsample of crested auklets (n=12) was fumigated with carbon dioxide (as described in Visnek and Dumbacher, 1999), and next dusted with pyrethrum (as described in Clayton and Walther, 1997) to evaluate the accuracy of the visual inspection method. The results are reported in Appendix 5.1. Ticks were identified as *Ixodes uriae* by Lance Durden and were deposited in the U.S. National Tick Collection (curated at Georgia Southern University) under accession number RML 123386. Lice were identified by Dale Clayton as belonging to the genera *Austromenopon, Quadraceps, Saemundssonia* and deposited into the frozen collections of the Price Institute for Phthirapteran Research at University of Utah in 2004.

RESULTS

Chemical emission rates differed among individuals but not between the sexes. Measurements from the field calibration obtained a mean peak area of 277,424,557 (S.E. = ± 4,586,885) from 3.0 ml of octanal passed into the volatile collection system. Applying this calibration, the average chemical emission for crested auklets was 5.7 μl octanal/50 min ± 0.42 (Peak Area = 529,800; S.E. = ± 38,800 or 7% sample mean).

There was a sevenfold difference between the highest and lowest chemical emissions. A male had the highest chemical emission at 19.9 µl/50 min (P.A. = 1,842,816), and the lowest chemical emission was also a male at 2.8 µl/50 min (P.A. = 262271). Male and female crested auklets did not differ in chemical emission rates, $t_{(0.05)2\text{-tailed}} = 0.44$, p = 0.66. The mean value for male crested auklets was 5.6 µl/50 min ± 0.52 (P.A. = 518900; S.E. = ± 48500, n=41) versus 6.0 µl/50 min ± 0.68 (P.A. = 557900; S.E. = ± 62600, n=16) for females. Measurements were also obtained for one subadult male and female with well developed ornaments. The male emitted 4.4 µl/50 min. (P.A. = 405974), and the female emitted 8.8 µl/50 min. (P.A. = 813506). Octanal was not detected in the control run when no bird was in the chamber.

Most birds had no ticks, despite a high abundance of *I. uriae* ticks on the colony surface. Prevalence of tick parasitism was very low. Only 2 out of 96 crested auklets examined by visual inspection methods had ticks. The individual with lowest octanal emission had 14 ticks attached between the rictal plate and eye on the left side of the face. All of these ticks were in the process of obtaining blood meals. The only other crested auklet that we saw parasitized by ticks had 2 attached to its face. This was just 2.1% of the population. Results for the visual inspection method were very similar to results for the combined methods of fumigation and dust ruffling (App. 5.1). Only one tick was found on 12 crested auklets by the combined methods of fumigation and dust ruffling, and this individual had not necessarily been successful at parasitizing its host. The low abundance of ticks on crested auklets was remarkable considering our own encounter rate with ticks in the colony. We noted 5-10 ticks on our pant legs per hour while sitting in

the auklet colony. We counted as many as 20 questing ticks (mostly nymphs) within small areas (0.37-0.91 sq. m) on the surface of large landing rocks where crested auklets alighted. Questing is the common term used to describe host seeking behavior in ticks

Visual inspection suggested that the prevalence and abundance of lice were low. Lice were found on 4 of the 96 birds inspected, an infection rate of 4.2%. Comparison of the visual inspection method with fumigation and pyrethrum dusting was in agreement with results from visual inspection (App. 5.1). Only one louse was found by fumigation and only one louse was found by dust ruffling on 12 crested auklets. No more than four lice were found on any bird, including specimens that were collected for dissections.

DISCUSSION

Differences in octanal emission rates may be related to the ability to produce odor. Higher emission rates probably represent a higher expenditure of lipid reserves since the aldehydes appear to be products of fatty acid synthesis (H. Douglas, unpubl. data). The male with the highest chemical "potency" in this study would have expended a minimum of 0.57 ml lipid per 24 hours in order to consistently maintain the same level of emissions. The male with the lowest chemical potency would have expended approximately seven times less lipid. Differences in emission rates might also be related to hormone levels and behaviors that help to perfuse odorants in plumage (H. Douglas, unpubl. data).

The prevalence of tick parasitism on adult crested auklets in this study (2.1%) was unexpectedly low considering the high abundance of ticks in the colony. It was also

much lower than what has been found in other subpolar seabirds where the parasitism of *Ixodes uriae* ticks have been studied. Infestations in subarctic colonial seabirds have been documented as high as 70% of the adult population (Barton et al., 1996), and intensity of *I. uriae* infestations in some subantarctic seabird colonies may be sufficient to cause mortality in adult King Penguins (*Aptenodytes patagonicus*; Gauthier-Clerc et al., 1998). Two species of Pitohui birds (New Guinea), also known for chemical defense, exhibited a lower than expected infection rate from ticks (3.1%, n=32) compared to other genera of passerines (Mouritsen and Madsen, 1994).

Higher rates of octanal emission in crested auklets may be associated with lower incidence of tick parasitism. Aldehydes could interfere with four stages of Ixodes tick parasitism – engagement, exploration, penetration and attachment (described in Kebede, 2004). Crested auklets thrust their bills and face into the scented nape feathers of conspecifics ("ruff-sniff" behavior) during courtship and social behavior (Jones, 1993b; Hunter and Jones, 1999; Hagelin et al., 2003; Jones et al., 2004). This behavior may provide an opportunity to evaluate chemical potency of prospective mates (Douglas et al. 2001, 2004), and it may also serve to distribute the odorant in plumage. Anointment of facial skin and plumage around the head and neck could help to deter ticks and interfere with the intraspecific chemical signaling that ticks use to locate attachment sites on the host (Sonenshine, 1985). In fact, aldehyde concentrations in crested auklet plumage are sufficient to reduce tick locomotion (H. Douglas, unpubl. data). Below some threshold of chemical defense crested auklets are likely to be more vulnerable to tick parasitism. In this study the crested auklet with the lowest chemical emission rate (half the population

mean) was parasitized by 14 ticks clustered together between the eye and rictal plate. Ticks often attach around the eyes of birds because the skin is thin, and the area cannot be preened by the host (Reed et al., 2003). Chemical potency and associated repellence of ectoparasites may be a basis for mutual sexual selection in crested auklets (Douglas et al. 2001, Douglas et al. 2004).

The louse infection rate among crested auklets at Big Koniuji I., as best could be determined, was 4.2%. Similarly, there was a very low infection rate at another monospecific colony of crested auklets. No lice were found on 80 adults at Talan I. (see Douglas et al., 2004, Chapter 2 for details). By contrast, the prevalence and abundance of lice were much higher in a mixed species colony that included both crested auklets and least auklets (*Aethia pusilla*) at St. Lawrence I., AK (Douglas et al., 2005a). Among these crested auklets there was a 100% infection rate (n=21), and the intensity of infection (adult + juvenile lice) ranged from 8 to 91 lice per bird (H. Douglas, unpubl. data). Numerical results are not directly comparable because data for Douglas et al. (2005a) were obtained by body washing, which is a more accurate method than visual inspection (Clayton and Drown, 2001). Nevertheless a qualitative difference can be inferred. To the extent that it has been evaluated, the prevalence of louse infections in crested auklets is higher at mixed species colonies than at monospecific crested auklet colonies.

This study reports on a novel research application for estimating chemical emission rates in crested auklets. The method has several advantages. Accurate quantitative estimates of chemical production and emissions can be obtained for live

vertebrates without harvesting tissues that might jeopardize the animal's fitness. The live specimen is isolated in a leak proof chamber supplied with a purified airstream. This eliminates the possibility of contamination from plant volatiles, insects, and naturally occurring materials. Flow rate is regulated and this is critical for determining rates. The quantitative measurements can be calibrated to known standards. The methods reported here could be applied to other vertebrates to study the relationship of chemical emissions to a range of studies including hormones, mate selection, reproductive behavior, or parasitism.

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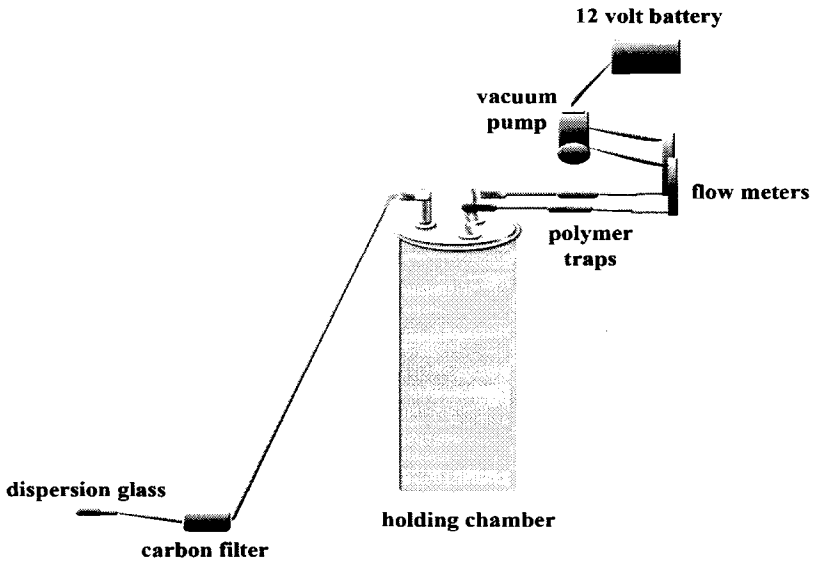

Figure 5.1 Volatile collection system for measuring chemical emissions. Regulated and purified air is pulled by battery-operated vacuum pump into glass reaction kettle containing live crested auklet. Volatile emissions are captured onto polymer traps that are placed in the exiting air stream.

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Appendix 5.1. Results of visual inspection methods versus combined methods of fumigation and dust ruffling, carried out on the same crested auklets, in sequential order.

Bird #	Visual Lice	Visual Ticks	CO2 Lice	CO2 Ticks	Pyrethrum Lice	pyrethrum Ticks
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	1	1	0
6	0	0	0	0	0	0
7	0	0	0	0	0	0
8	0	0	0	0	0	0
9	0	0	0	0	0	0
10	0	0	0	0	0	0
11	0	0	1	0	0	0
12	0	0	0	0	0	0

Chapter 6: Male ornament size is negatively correlated with corticosterone in colonial seabird[1]

Abstract We conducted the first study relating ornaments to corticosterone in the crested auklet, *Aethia cristatella*, a colonial seabird of Alaskan and Siberian waters. Crested auklets are one of the few avian species where both sexes employ a mutual ornament in both sexual and aggressive displays. Previous studies have shown that larger crest ornaments are associated with sexual attractiveness in both sexes, suggesting that the ornament is indicative of an individual's quality. However, no published study has established a relationship between crest size and condition. We hypothesized that physiological condition, as an indicator of individuals' competence at meeting energetic demands of reproduction, would correlate with crest size. We predicted that individuals with longer crests would be in better physiological condition. We used corticosterone concentrations (baseline corticosterone, BL, and adrenocortical response to acute stress, SR) as measures of physiological condition. Many captive and field studies have suggested that corticosterone is a reliable indicator of physiological condition, although this has not yet been demonstrated empirically in the crested auklet. We found that crest length predicted baseline corticosterone in males but not in females. This was also true for males when the cumulative ranks of baseline and stress response corticosterone were

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compared to crest length. In other words, males with longer crest ornaments had a stress hormone profile that is characteristic of birds with better physiological condition, while the opposite was true for males with short crest ornaments. Obtained results support viability models of sexual selection for males, in that the ornamental feather crest appears to communicate information about the relative quality of the individual to prospective mates and opponents. Sexual differences in the social and physiological costs of reproduction may explain the contrasting results in males and females.

Keywords: corticosterone, sexual selection, stress, body condition, allostatic load

6.1. Introduction

Secondary sexual traits, acquired during preparation for breeding, may reflect physiological condition at the time ornaments are acquired. Andersson (1994) noted that many male traits that are subject to female choice seem to develop in proportion to nutritional condition and health of the male, supporting the notion that secondary sexual traits can serve as indicators of mate quality. Some indicator models hypothesize that "costs" limit the expression of sexually selected traits. These costs may be expressed in a variety of fitness-related currencies including physiological and social costs, and risk of predation (Zahavi, 1975; Folstad and Karter, 1992; Berglund, 2000). In the case of physiological costs, Folstad and Karter (1992) proposed that a biochemical substance might serve as a mediator between immune and endocrine systems, limiting expression of extravagant traits. Hillgarth and Wingfield (1997) suggested that corticosterone might

serve as this mediator in birds, since it has been shown to influence both synthesis of sex hormones and expression of secondary sexual traits (Deviche et al., 1982).

We suspected that ornament expression may correlate with physiological condition. We looked for evidence of this in crested auklets, colonial seabirds of Alaskan and Siberian oceanic waters. Crested auklets are an example of mutual sexual selection. Males and females are nearly monomorphic in their ornamental traits, differing conspicuously only in the morphology of the bill (Jones, 1993a; Jones et al., 2000). Both sexes possess extravagant feather crest ornaments during the breeding season, consisting of stiff black feathers bunched together and arching forward from the forehead (Bédard and Sealy, 1984; Jones et al., 2000). Both sexes compete for mates in common social areas, and display preferentially to models with larger ornamental feather crests (Jones and Hunter, 1993; Jones and Hunter, 1999). Courtship, competition for mates, and social conflict over nesting crevices occurs prior to egg-laying for the breeding adults (Jones 1993b). Males and females tend to pair during courtship with mates of similar crest length, and crest length is associated with dominance in both sexes (Jones and Hunter, 1999). There is a high divorce rate between breeding seasons (approximately 25%; Zubakin and Zubakina, 1994). Females with short crests are more likely to divorce and seek males with longer crests in a subsequent breeding season (Fraser et al., 2004).

Although crest size is associated with social dominance and sexual attractiveness, previous studies did not establish a relationship between crest size and measures of intrinsic quality. Crest length was only weakly correlated (r^2=0.05) with body condition in female crested auklets, and there was no correlation between crest length and body

condition in males (Jones et al., 2000). Crest size did not predict future survival of individuals (Jones et al., 2004). However, survival may not accurately reflect intrinsic quality, particularly when the actual ages of individuals in the marked sample are unknown. Furthermore, natural selection may maximize reproductive success at the expense of individual longevity (Williams, 1957; Wilson and Daly, 1985; Nesse, 2001, 2005). Thus, to date there is no evidence that expression of ornaments is related to intrinsic quality of crested auklets.

We measured physiological condition in crested auklets as an indicator of relative intrinsic quality and compared this to measures of ornament expression. Corticosterone and adrenocortical response to acute stress were used as measures of physiological condition. Corticosterone (CORT) is secreted by the adrenal glands in response to energetic challenges modulated by the HPA axis and autonomic nervous system. The stimuli may be derived from predictable seasonal changes in energy needs or from unpredictable challenges that arise in the context of social conflict or changes in food availability (Wingfield et al., 1998; McEwen and Wingfield, 2003). Birds in relatively good physiological condition have better buffering against these challenges and this is manifested as relatively low CORT secretions and relatively low response to standardized acute stress. Birds in poor physiological condition have relatively high CORT secretions and relatively high response to standardized acute stress. The secretion of CORT is adaptive since it helps maintain energy balance, but it is also potentially maladaptive if secretions are excessive (Korte et al., 2005). Goymann and Wingfield (2004) evaluated literature values for glucocorticoid concentrations among dominant and subordinate

animals and predicted relationships between allostatic load and social dominance in different types of animal societies. Allostatic load is the physiological wear and tear that occurs in the process of maintaining a stable energy balance in the face of energetic demands, environmental challenges, and social conflict (McEwen and Wingfield, 2003). For example, allostatic load could accumulate from too frequent modulation of CORT levels or prolonged elevations of corticosterone. We tested predictions of sexual selection theory and Goymann-Wingfield's model in crested auklets. We predicted that larger crest size should correspond with better physiological condition, consistent with viability models that argue for a correlation between signal intensity and intrinsic quality (Zahavi, 1975; Hamilton and Zuk, 1982; Folstad and Karter, 1992; reviewed in Andersson, 1994).

6.2. Materials and Methods

6.2.1. Study Area

Research was conducted at Big Koniuji I., AK. This colony was reputed to be one of the largest breeding concentrations of crested auklets in existence (Townsend, 1913). However, this population declined precipitously from an estimated 300,000 individuals to just 30,000 by the mid-1970s due to predation by introduced red foxes (*Vulpes vulpes*) in 1916 and arctic foxes (*Alopex lagopus*) in 1925 (Bailey 1990). By the mid-1980's the population was estimated at just 6000 individuals, and despite the removal of fox in the mid-1980's (Bailey, 1990) had not increased significantly by 2002. In this study the population was estimated at 7000-10,000 individuals (H. Douglas,

unpubl. data). Colony size appeared to be limited by availability of suitable nesting habitat. There was a very large talus field below a glacial cirque. The talus field has become overgrown with tundra vegetation. Crested auklets only nest in the glacial cirque and only on the highest and steepest part of the talus slope.

6.2.2. Field Methods

We sampled birds after seasonal development of ornaments (which occurs earlier in spring). Breeding adult crested auklets were captured with noose carpets from early June to mid-July 2002. This spanned the period from early incubation to early chick rearing. The sample period was divided into three stages. Stage 1 spanned June 1-19, corresponding to early incubation. Stage 2 spanned June 20-July 4, corresponding to late incubation. Stage 3 spanned July 5-16, corresponding to early chick rearing. We judged phenology by monitoring the behavior of mated pairs, and we commenced sampling once the frequency of mated pairs decreased on the colony surface. Also, an adult female collected on June 3 had an egg follicle entering the oviduct. This helped to confirm our behavioral observations. The beginning of the chick-rearing period was determined by observing changes in the daily attendance pattern of adults at the colony and the audible vocalizations of recently hatched chicks. Blood samples (100 μl) were collected from femoral and alar veins at 0-3 minutes for baseline corticosterone (BL) and subsequently at 10 and 30 min for measurement of the adrenocortical response to acute stress (SR). Samples collected within 3 min after inducing handling stress represent baseline levels, because it usually takes more than 3 min for corticosterone levels to increase (Romero and Reed, 2005). This was also true for our research, since the time of bleeding within

the first 3 minutes after capture had no significant effect on baseline CORT measurements (R^2=0.01, F=0.59, *df*=58, *P*=0.12). Blood samples were placed on ice immediately after collection, and plasma was separated from red blood cells by centrifugation within 12 hours of collection and stored frozen at -20°C until radioimmunoassay analyses (for details see Wingfield and Farner, 1975; Wingfield et al., 1992). The concentrations of CORT in plasma samples were measured from 20 µl of plasma. Steroid concentrations were measured in duplicate for each sample after extraction in 4 ml of dichloromethane. Recovery values (ranging from 73 to 100%, mean 85.8%) following extraction were used to adjust assayed concentrations of steroids. All samples were analyzed in a single assay; the intra-assay variance was 2% and assay sensitivity was 7.8 pg/tube.

Birds were measured for body condition and ornamental traits, and banded with numbered metal bands (USFWS) in between bleedings. Body condition was characterized as mass-skeletal dimensional ratio. Morphological measurements were taken with dial calipers (± 0.05 mm) using methods similar to those described in Jones et al. (2000). Observer precision was compared several times across the study period to ensure reproducibility of measurements. Breeding status was confirmed by the presence of a full brood patch. Sex was judged according to bill characteristics, a morphological trait that reliably separates males from females (Jones, 1993a). Ambiguous cases where sex could not clearly be determined were excluded from analyses where only males or females were evaluated. Birds were placed in opaque bags when they were not being handled.

6.2.3. Data Analysis

We evaluated relationships between ornamental traits, physiological condition, and body condition with SAS (Vers. 9.1), SPSS 14.0 and ProStat (Vers. 3, Poly Software International). One-Way ANOVA was used to test for seasonal trends in physiological condition. Correlation matrices were produced to examine univariate relationships among body condition, ornaments, and corticosterone concentrations. The following variables were tested in correlation matrices. Body condition, a proxy for endogenous reserves, was evaluated by a mass-dimensional ratio ($MASS/TARSUS^3 \times 100$). This measurement has been used as an index of body condition in other studies of crested auklets (Jones et al., 2000). Ornamental traits were crest length, number of crest feathers, length of the right auricular plume (both sexes have a white auricular plume extending from behind the eye), and height of the right accessory bill plate (rictal plate). Current physiological condition was quantified by baseline (BL) concentration of corticosterone in the blood at the time of capture (0 - 3 min after capture). Parameters that correlated significantly with BL CORT were also examined by linear regression. Individuals were also compared according to cumulative scores for ranks at baseline, CORT 10 min, and CORT 30 min. This measurement combines BL CORT and stress response (SR) as an index of allostatic load. Applications of the stress series protocol developed by J. Wingfield and colleagues have shown that the shape and magnitude of the stress response profile are indicative of the relative sensitivity of the adrenocortical axis. Individuals with low baseline and a shallow, flat rise in CORT rank lowest in cumulative score. Individuals with a high baseline and rapid and steep increase in CORT rank highest.

Applications of the stress series protocol developed by J. Wingfield and colleagues have shown that the shape and magnitude of the stress response profile are indicative of the relative sensitivity of the adrenocortical axis (Wingfield, 1994; Wingfield et al., 1994a, 1994b, 1997). A rapid and steep increase in plasma concentrations of corticosterone indicates higher sensitivity of the adrenocortical response to acute stress, while, in comparison a shallow, relatively flat rise in corticosterone indicates that an animal has better physiological buffering against acute stress (Wingfield et al., 1997). We also examined rate of corticosterone secretion. RATE 1-2 and RATE 2-3 were the rates of increasing corticosterone concentration between the 1st and 2nd bleedings and between the 2nd and 3rd bleedings, respectively. Rate was calculated in the following manner:

$$Rate1-2 = (Cort_{time2} - Cort_{time1})/(Time2 - Time1)$$

for RATE 1-2 and similarly for RATE 2-3, with appropriate substitution.

6.3. Results

6.3.1. Seasonal effects

There were no seasonal differences in baseline (BL) corticosterone concentrations and stress response (SR) parameters (P = 0.11 to 0.94; Table 1). The same was true for body condition (P = 0.55; Table 2). There were no differences between males and females with respect to BL CORT ($t_{0.05(2)51}$ = 1.02, P=0.31), body condition ($t_{0.05(2)82}$ = 0.92, P=0.36), or SR CORT (CORT 10 Min, P=0.21; CORT 30 Min, P=0.67). The rate of corticosterone secretion between first and second bleedings (RATE 1-2) was higher In females than males (1.88 versus 1.25 ng/ml/min; $t_{0.05(2)51}$ = 2.23, P=0.03).

6.3.2. Sex effects

6.3.2.1. Corticosterone levels vs. body condition

BL CORT was not correlated with body condition in males (P=0.5). BL CORT was negatively correlated with body condition in females (r = -0.62, P = 0.008, n=17; Fig. 1). There was one outlier with high CORT, but the relationship was similar after the outlier was removed (r = -0.60, P = 0.01, n=16). Applied in a linear regression model, BL CORT explained 38% of variation in body condition for females ($F_{(1,15)}$=9.37, P=0.008) yielding the following regression equation:

Body Condition = .788 - 0.008(BL CORT)

The correlation between body condition and cumulative rank for combined scores of BL and SR approached significance in females (r_s= -0.48, P=0.059, n=16), but not in males (r_s= 0.27, P=0.13, n=31).

6.3.2.2. Corticosterone levels vs. ornaments

Baseline corticosterone concentrations (BL CORT) correlated negatively with length of the crest ornament in males (r = -0.59, P<0.001, n = 35; Fig. 2) but not in females (P=0.63). Applied in a linear regression model, BL explained 35% of variation in male crest length ($F_{(1,33)}$=17.85, P < 0.001), yielding the following regression equation:

Crest Length = 45.01 – (0.54 * BL)

The relationship between crest size and BL in males also was evident in combined ranks of BL and SR (BL + 10 min CORT + 30 min CORT). Males with longer crests had a lower combined score (r_s = -0.44, P= 0.01, n=31). Number of crest feathers also correlated with BL CORT in males (r= -0.36, P=0.038, n=34).

6.3.2.3 Ornaments

In females, height of the rictal plate approached a significant correlation with BL CORT (r= -0.50, P=0.058, n=15). No ornamental traits correlated with body condition in males or females (P=0.19 to 0.94). The auricular plume did not correlate with any parameter in either sex (P=0.31 to 0.96).

6.3.2.4. Morphological traits

In males, length of the bill was positively correlated with BL CORT (r=0.35, P=0.038, n=35) and crest length (r_s=0.28, P=0.036, n=55). Depth of the bill was positively correlated with mass (r=0.31, P=0.02, n=55) and height of the rictal plate (r=0.37, P=0.008, n=52). Height of the rictal plate was also strongly correlated with mass (r=0.47, P<0.001, n=52). Mass was not correlated with BL CORT (r=0.02, P=0.99, n=36). Mass and tarsus scaled together (r=0.49, P<0.001, n=55).

In females, bill depth was positively correlated with length of the bill (culmen) (r=0.38, P=0.048, n=28) and height of the rictal plate (r=0.56, P=0.004, n=25). Mass approached a significant negative correlation with BL CORT (r=-0.47, P=0.055, n=17). Mass and tarsus scaled together (r=0.41, P=0.028, n=29).

6.4. **Discussion**

There were no seasonal differences in baseline corticosterone (BL), adrenocortical response (SR), or body condition at Big Koniuji (Table 1), suggesting that nutritional stress did not change across the breeding season. Baseline corticosterone and adrenocortical response values generally reflect local environmental conditions

(Pravosudov et al., 2004). Seasonal trends in these parameters are often evident at subarctic seabird colonies, including crested auklet colonies (e.g., Pribilof Is., Kitaysky et al., 1999a; Kitaysky et al., 2002). Baseline corticosterone (BL) has been used as an indicator of current physiological condition at Alaskan seabird colonies, and adrenocortical response to acute stress (SR) has been used as an indicator of recent physiological condition (Kitaysky et al., 1999a). Experimental studies have demonstrated that nutritional limitation in seabirds leads to elevation of baseline corticosterone, and sustained nutritional limitations also elevate adrenocortical response to acute stress (Kitaysky et al., 1999b, 2001, 2003, 2005a,b). In nature, nutritional limitations due to declining food availability can be manifested as increased BL and SR across the breeding season (Kitaysky et al., 1999a). Considering the former size of the Big Koniuji auklet colony (~300,000) and its limited population at present (~7000-10,000), it is possible that food resources within the crested auklet's foraging range are underexploited at this colony. The lack of seasonal differences in stress hormones and body condition made Big Koniuji useful for studying correlates of ornaments since the confounding effects of nutritional stress can be assumed to be low.

Previous studies have shown that crested auklets prefer mates with longer crests (Jones and Hunter, 1993; Jones and Hunter, 1999; Fraser et al., 2004), and this study shows that males with larger crest ornaments also have lower baseline corticosterone and lower stress profiles. It is logical to interpret these two results as the following -- males with larger crest ornaments are more likely to have higher reproductive success and survival. In other species of birds and vertebrates, increased levels of corticosterone are

negatively correlated with reproductive performance and in some cases with post-breeding survival (Lanctot et al., 2003; Love et al., 2004; Romero and Wikelski 2001). Thus, we conclude that the crest ornament in crested auklets does provide a signal of mate quality.

As we expected, corticosterone levels were correlated with ornaments and body condition of crested auklets, reinforcing the idea that this steroid hormone is a useful measure of physiological condition. Females and males face different trade-offs in optimizing their fitness, and this probably causes them to invest their resources in different ways. Females are smaller and lighter than males, and invest endogenous resources into egg synthesis. Male crested auklets devote more of their resources to agonistic conflict aimed at guarding their mate, nest site, and offspring (Jones and Hunter, 1999; Fraser et al., 2002). These differences may explain sex-dependent patterns in body condition and corticosterone secretion. Body condition, a proxy for endogenous fat energy reserves, is often negatively correlated with corticosterone concentrations and thus energy balance in birds (Wingfield, 1994; Wingfield et al., 1994a,b, 1997). However, in our study males and females differed in this relationship. Female body condition was negatively correlated with corticosterone concentrations, but this was not the case in males (Fig. 1). Females also had a higher initial rate of corticosterone secretion (RATE 1-2) than males following capture, perhaps because they were more dependent upon endogenous reserves for maintenance. Males probably pay higher social costs for breeding than females. Jones and Hunter (1999) found that agonistic interactions in males were more likely to progress to physical combat, whereas agonistic

interactions in females were more likely to be resolved by signaling with the crest ornament. Interestingly, Jones et al. (2000) noted that there was greater variability in the crest size of females than males, and they suggested this was probably related to the importance of the female's crest for mediating conflict (Jones and Hunter 1999). Stronger intrasexual selection for aggression in males is also suggested by the males' bill, which is strongly hooked and used in fighting (Jones, 1993a). Males with larger crests tended to have larger bills, suggesting that a large armament is associated with a larger ornament in this species. This is consistent with the theory that evolution of ornaments was linked to the evolution of armaments used in male-male combat (Berglund, 1996). Bill size was positively correlated with corticosterone in males and also with higher androgens (H. Douglas, unpubl. data), suggesting that males with larger bills may be more aggressive and involved in more fights. Evolution of the crest ornament may have been favored because it causes the head and the bill armament to appear larger.

Male crested auklets fight over nest sites, and their corticosterone levels may reflect social hierarchy, competition for resources, and competence at meeting energy demands. The crest ornament mediates conflict and in this sense the ornament functions as a badge of social status (Jones and Hunter, 1999). Such badges arise in the interactions of animal societies because they increase fitness in the bearers, and badges accomplish this by reducing the incidence of direct combat (Darwin, 1871; Rohwer, 1982). Signaling with larger status badges brings on more challenges from opponents with higher status (Balph et al., 1979; Møller, 1987; Maynard Smith and Harper, 1988), and this can reduce the quality of male parental care (Quarnström, 1997). Male crested

auklets provide significant parental care (Fraser et al., 2002). Increased physiological costs from male-male competition could reduce the quality of parental care and thereby reduce fitness. Theoretically, females' reproductive decisions should benefit from assessing males' badges of status (Johnstone and Norris, 1993), and this is the case in crested auklets. Female crested auklets divorce males with shorter crests in preference for males with longer crests (Fraser et al., 2004). The signaling system in crested auklets is rendered honest if individuals with longer crests are better able to meet the energetic costs and social costs of reproduction, while lower quality individuals that signal deceptively incur increased physiological costs.

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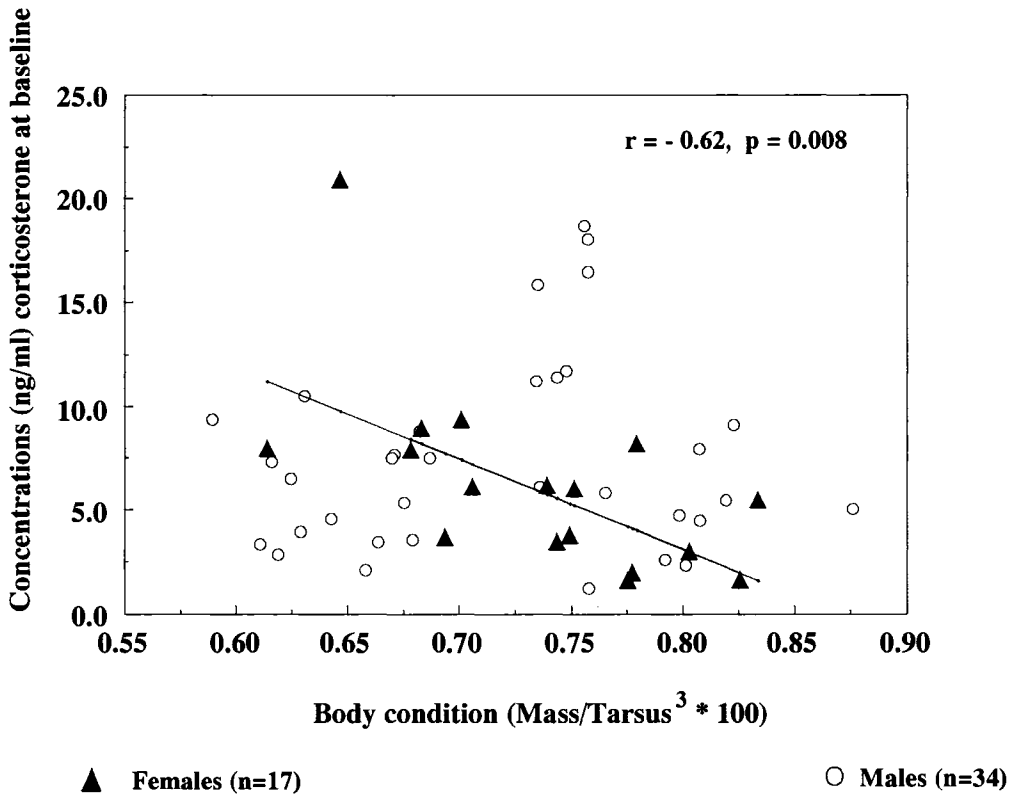

Figure 6.1. Body condition in females (but not males) correlated negatively with baseline corticosterone concentrations.

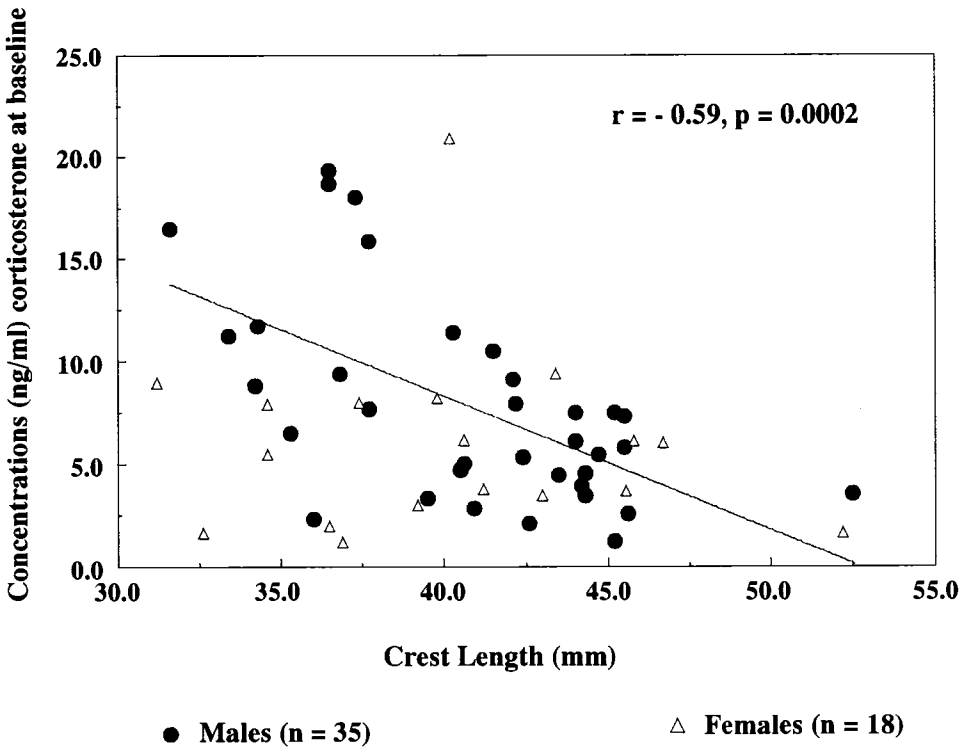

Figure 6.2. Length of the male crest ornament correlated negatively with baseline corticosterone.

Table 6.1 Seasonal patterns of corticosterone and adrenocortical response in breeding adult crested auklets at Big Koniuji Island, Alaska in 2002

Sampling Stage	Baseline CORT	CORT 10 min	CORT 30 min
	ng/ml ± SD (*n*)	ng/ml ± SD (*n*)	ng/ml ± SD (*n*)
Early incubation	6.42 ± 4.44 (27)	17.92 ± 8.52 (27)	34.0 ± 10.5 (21)
Range	*1.2 – 19.36*	*5.75 – 41.69*	*15.55 – 49.49*
Late incubation	6.56 ± 4.34 (16)	19.0 ± 8.03 (15)	32.77 ± 9.4 (14)
Range	*1.2 – 18.07*	*8.30 – 40.77*	*16.81 – 50.26*
Early chick rearing	8.86 ± 5.18 (17)	17.68 ± 9.22 (16)	30.59 ± 10.46 (16)
Range	*1.66 – 20.93*	*10.32 – 48.9*	*13.22 – 55.14*

Statistical Results – One Way ANOVA

Baseline CORT	$F_{(2,59)} = 2.30$, P=0.11	Males $F_{(2,35)} = 0.25$, P=0.78
		Females $F_{(2,16)} = 1.09$, P=0.36
CORT 10 min	$F_{(2,57)} = 0.066$, P=0.94	Males $F_{(2,35)} = 0.43$, P=0.65
		Females $F_{(2,16)} = 0.34$, P=0.72
CORT 30 min	$F_{(2,50)} = 0.347$, P=0.71	Males $F_{(2,30)} = 0.13$, P=0.88
		Females $F_{(2,15)} = 0.02$, P=0.98

Table 6.2 Seasonal variation in body condition of breeding adult crested auklets at Big Koniuji Island, Alaska in 2002

Sampling Stage	Mass/Tarsus^3 * 100	Range
	x ± SD (*n*)	
Early incubation	0.713 ± 0.095 (41)	.555 – 1.01
Late incubation	0.733 ± 0.072 (22)	.589 – .867
Early chick rearing	0.709 ± 0.066 (26)	.614 – .876

Statistical Results

One-Way ANOVA $F_{(2, 88)} = 0.61$, $P = 0.55$

Males $F_{(2,54)} = 0.72$ P=0.49

Females $F_{(2,28)} = 0.72$ P=0.50

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Chapter 7: Seasonal patterns in steroid hormones and octanal emissions of the crested auklet (*Aethia cristatella*)[1]

Abstract

We studied seasonal patterns of steroid hormone secretions and volatile chemical emissions in a small colony of crested auklets (*Aethia cristatella*). Crested auklets emit a citrus-like odorant during the breeding season. The aldehyde constituents of the odorant disappear from plumage late in the breeding season at about the same time that plumage ornaments senesce and daily social periods cease. We hypothesized that expression of the chemical odorant is facilitated by steroid hormones, like other secondary sexual traits in birds. We captured male and female crested auklets and collected blood samples for radioimmunoassay of steroid hormones. Auklets were placed in a purified air stream, and volatile chemical emissions of auklets were captured by suction onto polymer traps. The elutions of polymer traps were analyzed by GC/MS. In both sexes, progesterone was relatively high during early incubation, declined during late incubation, and increased during early chick rearing. Octanal emissions were highest during the estimated dates of egg hatch and the first week of chick rearing. Octanal emissions were correlated with progesterone in males. No seasonal patterns were detected in androgens and estrogens, and these hormones were not detected in all breeding adults.

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Keywords: Chemical signaling, progesterone, steroid hormones, aldehydes, courtship

Introduction

Steroid sex hormones facilitate expression of secondary sexual traits and reproductive behaviors in birds (Balthazart, 1983; Gonzalez et al., 2001; Ketterson and Nolan, 1992). They provide a general signal for increased reproductive behavior that is translated in different ways at specific targets (Balthazart and Ball, 1993; Ketterson and Nolan, 1999). For example, quail copulation behavior depends upon aromatization of testosterone (T) into estradiol (E_2) at the preoptic medial nucleus, while crowing depends upon enzymatic reduction of T to 5-alpha-dihydrotestosterone (DHT) in the nucleus intercollicularis (Balthazart and Ball, 1993). T enhances expression of secondary sexual traits and behaviors associated with acquiring breeding territories and mates (Arnold, 1982; reviewed in Ball et al., 2002; Guillermo, 2001; Ketterson et al., 1992; Nowicki and Ball, 1989; reviewed in Roberts et al., 2004; Wingfield et al., 1987). Both T and E_2 influence nest-building behavior (Cheng and Silver, 1975; Hinde et al., 1974; Logan and Carlin, 1991). The functions of the sex hormone progesterone (P_4) are not well understood in birds, but P_4 may modify or complement the effect of other steroids (Belle et al., 2003; Logan and Wingfield, 1995).

There is considerable evidence for chemical communication in mammals, reptiles, and amphibians but relatively few examples of chemical communication in birds. Mammalian odors important in communication are derived from diverse sources (e.g., specialized glands, urine, vaginal secretions) and function in a variety of contexts, including individual recognition, social status, territory marking, mate choice, and

reproduction (Albone, 1984; Agosta, 1992; Kruuk et al., 1984; Rasmussen, 2001; Sun and Müller-Schwarze, 1998; Smith et al., 2001). For example, pheromones emitted by male sheep (*Ovis* sp.) and goats (*Capra* sp.) activate reproductive function in females (Chemineau, 1987; Martin et al., 1980; Ott et al. 1980; Shelton, 1960). Similarly, there are notable examples of chemical communication in amphibians and reptiles that help to mediate reproduction. Amphibian pheromones are peptides secreted by skin glands, synthesized under regulation by hormones such as androgens and prolactin, and many of these pheromones play a role in reproduction (reviewed in Rajchard, 2005). For example, male plethodontid salamanders deliver a nonvolatile contact pheromone from the male's submandibular gland to the female's nares that increases the female's receptivity to courtship and copulation (Houck and Reagan, 1990; Houck et al., 1998; Rollmann and Houck, 1999). Chemical communication has been well documented in lizards (Mason, 1992) discriminating between chemical odors of "self" versus "others" (Graves and Halpern, 1991), for territorial marking (Alberts, 1992), for discrimination of individuals (Cooper, 1996), and for sexual recognition (Cooper et al., 1996). Among red-sided garter snakes (*Thamnophis sirtalis parietalis*) there are population differences in the chemical composition of the female sexual attractiveness pheromone, and males prefer the female chemical cue of their own population (Lemaster and Mason, 2003). Both amphibians and reptiles sense chemical signals with vomeronasal organs, in contrast to birds, which lack such organs and are dependent upon olfaction for chemical sensing. Among all the classes of vertebrates, chemical communication is the least well documented in the Class Aves (Agosta, 1992; Roper, 1999). Uropygial secretions of

female mallard ducks (*Anas platyrhynchos*) and Procellariiform seabirds may have some importance in chemical communication. Female mallards increase production of diester waxes in their uropygial gland (preen gland) during the breeding season, and this biochemical change appears to influence male sexual behavior (Balthazart and Schoffeniels, 1979; Jacob et al., 1979). Seasonal changes in odor production coincident with breeding have also been noted in some Procellariform seabirds (Thibault and Holyoak, 1978). Olfactory nest recognition and mate recognition were documented in some species of this group (Bonadonna and Nevitt, 2004; Minguez, 1997).

Odor may also mediate social relations in crested auklets (*Aethia cristatella*). Both sexes emit a citrus-like odor during the breeding season. Saturated and monounsaturated aldehydes, 6-12 carbons in length, are the dominant constituents of the crested auklet odorant (Douglas et. al, 2001, 2004; Hagelin et al., 2003). In T-maze experiments crested auklets avoided a musk odor, similar to the scent of mammalian predators, showing that they do respond to scent (Hagelin, et al., 2003). In a separate trial crested auklets spent more time in the side of the T-maze scented with synthetic odor constituents of their own species; however, there was no chemical control in the other side of the maze (Hagelin, et al., 2003). In a follow-up field experiment, crested auklets were more attracted to life-like taxidermic mounts with augmented concentrations of octanal and Z-4-decenal (Jones et al., 2004). Male crested auklets approached scented male models more closely ($p=.04$) and for longer duration than control models ($p=.03$). Similarly females approached scented male models more closely ($p=.02$) but not for longer duration ($p=.75$) (Jones et al., 2004).

We suspected that hormones mediate odorant production in crested auklets. We hypothesized that steroid hormones facilitate expression of crested auklet's chemical odorant. We predicted that variation in chemical emissions would correspond with variation in concentrations of circulating steroid hormones among breeding adult crested auklets. In order to test this hypothesis we measured individual variation in chemical emissions and circulating concentrations of steroid hormones.

Methods

Field Methods

Research was conducted at a small colony of crested auklets on Big Koniuji I., AK in 2002. The colony is situated in an ancient glacial cirque at 243 m elevation on a mountain overlooking Yukon Harbor. Sampling occurred from June 4 to July 16. This corresponded with onset of egg-laying to early chick rearing. We monitored behavior at the colony and commenced sampling after the frequency of mated pairs decreased on the colony surface. Crested auklets nest deep in rock talus on the steep slopes of the cirque. We searched the colony with flashlights, but no nests were visible. Three adult males taken May 28-31 had mature testes and developing brood patches (x=18.25 ± 0.95 mm length, compared to 17.1 ± 2.5 reported in Jones, 1993a). An adult female taken on June 3 (ovary=10.6 mm x 7.4 mm) had a developing egg follicle entering the oviduct. These data helped to support the inference from behavioral observations that egg-laying commenced in early June. This was consistent with what is expected for this latitude based on studies in the Aleutian Is. (Fraser et al., 1999). Egg laying in crested auklets tends to be highly synchronized within colonies in the northern part of their range (Gall et

al., 2006) but less so in the southern part of their range (Fraser et al., 1999). Avian predation upon auklets was intense at Big Koniuji. There were many avian predators hunting the small population of crested auklets (7000-10000 individuals in 2002, H. Douglas, unpubl. data). The location of the colony in a high elevation glacial cirque made it more difficult for crested auklet flocks to maneuver and evade these avian predators (peregrine falcon, *Falco peregrinus*; American bald eagle, *Haliaeetus leucocephalus*). The flock behavior of crested auklets at Big Koniuji was more coordinated and more synchronized than at other colonies, probably as a result of the predation threat (H. Douglas, unpubl. data). Breeding phenology at Big Koniuji may have been more synchronized as a result because late breeding could increase the likelihood of predation, while synchronization of breeding with the population could reduce predation risk. We recognized egg hatching and the beginning of the chick-rearing period by a conspicuous change in the daily attendance pattern of adults at the colony and the audible vocalizations of recently hatched chicks. During the incubation period, crested auklets aggregated as a large flock on the water in Yukon Harbor at about 0700. Flocks usually tried to enter the cirque between 0745 and 0900. On June 30, crested auklets amassed at 0700, but did not enter the colony until 1120. From that date on the synchronicity of colony attendance began to decrease. There were waves of smaller flocks during the day, and the colony attendance period extended into the afternoon. We heard the first vocalizations of crested auklet chicks on July 8. We surmised that those chicks could have hatched from eggs several days earlier. Our best estimate for a hatch date was July 5. This agreed with an average incubation period of 35

days (Fraser et al., 1999) and an egg-laying date in early June. Overall this was similar to the breeding phenology noted for crested auklets in the Aleutian Is. (Fraser et al., 1999).

Crested auklets were captured with noose carpets as they landed on the colony surface. Breeding adults arrived all at once in large flocks. Each bird was measured, banded, and sampled for corticosterone and sex hormones. Breeding status was confirmed by the presence of a full brood patch. Two subadults were also sampled for a descriptive comparison. We judged that these were subadults based upon worn brownish flight feathers (also noted by Bédard and Sealy, 1984: Jones et al., 2000), lack of a brood patch, and a relatively small crest ornament. Sex of all birds was judged according to bill characteristics; this is one morphological trait that reliably separates males from females (Jones, 1993b). Blood samples (400-500 μl) for androgens and estrogens were collected between 4 and 8 min. after capture. Blood plasma was separated from red blood cells by centrifugation within 12 hours of collection and frozen at -20°C until radioimmunoassay analyses (for details see Wingfield and Farner, 1975; Wingfield et al., 1992).

Hormone Assays

All androgens and estrogens were separated using column chromatography in a single assay and analyses were performed according to methods described in Wingfield and Farner (1975) and Wingfield et al. (1992). Approximately 110 μl of plasma were equilibrated with 2000 cpm of radio-labeled hormones to assess percent recovery. Samples were assayed in duplicates after double extraction, first with 5.0 ml distilled dichloromethane, followed by 4.0 ml ethyl ether. After extraction, samples were dried down under continuous nitrogen flow in a water bath at 40°C, reconstituted in 0.5 ml

10% ethyl-acetate in iso-octane, and added to columns of diatomaceous earth, with a water trap and glycol phase. Progesterone (P_4) was eluted in the first fraction, 2% ethyl-acetate in iso-octane, dihydrotestosterone (DHT) was eluted in the second fraction, 10% ethyl-acetate in iso-octane, testosterone (T) was eluted in the third fraction, 20% ethyl-acetate in iso-octane, and estradiol (E_2) was eluted in the fourth fraction, 40% ethyl-acetate in iso-octane. Mean recoveries were: P – 54%, DHT – 40%, T – 65%, E2 – 54%. Sensitivities of assays were: P – 3.9 pg/tube; and DHT, T, and E2 – 1.95 pg/tube. The intra-assay coefficients of variation were <3%.

Measuring Chemical Emissions

Detailed methodology has been reported in Chapter 5 (Douglas, in press) and will be described only briefly here. After sampling and measurements each bird was placed in a glass reaction kettle, and volatile emissions were collected in a purified air stream for 50 min. A filtered air stream was passed through a glass chamber (4 L Pyrex reaction kettle). The outgoing air stream was split into two custom-designed glass collection traps, packed with chromatographic polymers. All tubing and fittings were glass or Teflon. Air flow through each collection trap was regulated at 500 ml/min by Gilmont flow meters hooked up to a battery-operated vacuum source. After volatile collection the birds were released, and the traps were eluted with 2.0 ml of methanol. The elution was collected in borosilicate glass vials sealed with Teflon-lined caps and a vapor seal. The volatile collection systems were placed under a large rock overhang; this helped maintain temperature at 6-10° C during volatile sampling. Blanks controls with no birds in the chambers were run once at the end of the season. The volatile collection system was

calibrated in the field with synthetic octanal passed through a bubbler, placed in-line, upstream of the volatile collection chamber.

Chemical analysis was carried out by gas chromatography-mass spectrometry using a HP5890 Series II Gas Chromatograph equipped with a 20 m x 0.25 mm, 5% phenyl siloxane column (Alltech), and a HP5972 Series Mass Selective Detector. Octanal was selected as an index of chemical potency because it is consistently the most abundant constituent in the crested auklet odorant (40%, Douglas et al., 2001; Douglas et al., 2004). Retention time and ion abundances were obtained in EI mode from standard (99% Octanal, ACROS Organics, C.A.S. 124-13-0), and results were consistent across five replicates at different concentrations. Quality control was ensured by the inclusion of blanks, duplicates, internal standards, augmented standards, and calibration standards run in sequences at the frequency of 5-10% of total samples. Standards were made by serial dilutions in methanol (ACROS Organics HPLC grade). Undecenal (97% Undecyclenic Aldehyde, ACROS Organics, C.A.S. 112-45-8) was used as an internal standard, and this internal standard was added to all samples. Instrument variability of the GC/MS was accounted for by calibrating response factors of the target analyte and the internal standard. Quantitative measurements, obtained from field calibration with synthetic octanal and bubbler, were used to calculate emission rates for the birds. Results were reported as µl/50 min. Detailed information about chemical analysis is provided in Chapter 5.

Data for chemical emissions and hormones were analyzed using SPSS 14.0 and ProStat (Vers. 3, Poly Software International). Hormone data were examined in relation

to three stages of breeding. Stage 1 spanned June 1-19, corresponding to estimated period for early incubation. Stage 2 spanned June 20- July 4, corresponding to estimated period for late incubation. Stage 3 spanned July 5-16, corresponding to early chick rearing. Chemical data were examined in relation to 10-day intervals.

Results

Seasonal Patterns in Hormones - Progesterone (P_4) was the only sex hormone that was detected consistently in all samples, and it showed a seasonal dynamic in both sexes. The seasonal pattern of P_4 was evident as a broad U-shaped curve with the highest concentrations occurring during chick rearing (Fig. 7.1). P_4 levels differed between stages (ANOVA $F_{(2,59)}=9.99$, $p<.001$, n=62). P_4 was higher in Stages 1 and 3 ($x=1.92 \pm 0.15$, n=23; $x=2.64 \pm 1.34$, n=19) than in Stage 2 ($x=1.32 \pm 0.56$, n=20; Dunnett T3 $p< 0.05$ and <0.005, respectively). The overall range for P_4 was 0.33-6.22 ng/ml. The seasonal dynamic of P_4 was best described by a cubic regression ($R^2=0.41$, $p<0.001$, n=62). There was no detectable seasonal pattern in androgens (Fig. 7.2; $0.97 > p > 0.46$, T and DHT). Estradiol (E_2) may have decreased over time ($r=-0.5$ $p=0.1$), but it was only detected in 18% of samples (11 of 62) at low concentrations (0.07-2.23 ng/ml). T (0.05-3.49 ng/ml) was detected in 44% of samples, and DHT (0.10-1.52 ng/ml) was found in 44% of samples. There were no differences in the concentrations of sex steroids between males and females (p values ranging from 0.16 to 0.92). The two subadults sampled had similar levels of P_4 compared to adults. One subadult female sampled on July 7 had 1.28 ng/ml P_4, and one subadult male sampled on July 8 had 0.06 ng/ml P_4. Only the subadult

female had detectable levels of T (0.06 ng/ml), and neither subadult had detectable levels of E_2 or DHT.

Seasonal Pattern of Chemical Emissions - Comparison of 10-day intervals showed that octanal emissions differed across the sample period (ANOVA $F_{(4,53)}$=3.64, p=.01, n=54). The highest mean value (7.6 µl/50 min ± 0.85, n=16) occurred near the estimated dates of egg hatching between June 30 and July 10 (Fig. 7.3). This was greater than at 0-10 days (4.2 µl ± 0.49, n=8; p=0.02) and 10-20 days (4.3 µl ± 0.29, n=17; p=0.02) according to Dunnett's T3 post-hoc test. Octanal emissions were similar between males and females in terms of mean values (5.6 ± 0.52 vs. 6.0 ± 0.68 µl/50 min; $t_{(0.05)2\text{-tailed}}$ = 0.44, p = 0.66) and ranges (2.8–19.9 vs. 3.3–13.8 µl/50 min). Two subadults sampled late in the breeding season had octanal values (male =4.4, female=8.8 µl/50 min) similar to breeding adults.

Hormones and Chemical Emissions – Octanal emissions were correlated with progesterone in males (r = 0.36, p = 0.048, n=31) but not in females (p=0.44). The individual with the highest chemical emissions (19.9 µl/50 min) and highest P_4 (6.22 ng/ml) had 4 times more octanal (5.0 ± 0.2) and two times more P_4 (2.8 ± 0.47) than other auklets sampled during the same interval (40-50 days, n=7). At the other tail of the distribution, the male with the lowest chemical emission also had the lowest P_4 in Stage 1 (1.01 ng/ml), and the third lowest P_4 for the entire sample of breeding adults. Octanal was not correlated with other sex steroids in males or females (p ranging 0.19-0.87 and 0.56-0.72, respectively).

Discussion

Seasonal patterns in hormones and chemical emissions suggest that production of the crested auklet's odorant could be facilitated by progesterone. Octanal correlated positively with progesterone in males. The average values of progesterone and octanal increased at the time of egg hatching and early chick rearing. The male with the highest values of octanal also had the highest progesterone. The lowest values of octanal occurred in the bird with the lowest progesterone for Stage I, and this was also the third lowest progesterone overall. There are three factors to consider in interpreting this relationship between octanal and progesterone. First, there may be a time lag between changes in circulating hormone concentrations and changes in odor production. Second, progesterone could be a precursor for another hormone that more directly facilitates odor production. Progesterone is a metabolic intermediate in the biosynthesis of most steroid hormones, and it is also a sex hormone in its own right (Bentley, 1998, p. 70; Bobes et al., 2003). Progesterone could be converted into androgens and estrogens by secretory cells in the dermis, and those steroids are known to be influential in expression of chemical scents among vertebrates (Bentley, 1998, p. 308). Third, anointment behavior helps distribute aldehydes in plumage, and so the amount of chemical emissions is probably dependent in part on crested auklet behavior (Chapter 8, H. Douglas, unpubl. data). Progesterone could help facilitate expression of the crested auklet's odorant in the sense that it makes the processes of odor synthesis more likely to occur at a higher rate.

It is possible that progesterone and octanal may be important to parental care. Increases in concentrations of progesterone and emissions of octanal co-occurred with the

onset of egg hatching and chick rearing. The function of progesterone in birds is not well understood, but it appears to influence the reproductive cycle, modify the effects of androgens, and modify behavior. In other avian species, increased levels of progesterone have been associated with ovulation (Bentley, 1998, p. 429; Fraps, 1955; Furr and Smith, 1975; Nakada et al., 1994), a reduction in aggressive behaviors following courtship (Belle et al., 2003), and brood rearing (Logan and Wingfield, 1995). Increased progesterone may help modify the behavior of crested auklets towards their chicks. Crested auklets brood their chicks during the first week to help with thermoregulation, and to guard the chick against attacks by other adult crested auklets (Fraser et al., 2002, Piatt et al., 1990). Chemical emissions by adult crested auklets could help defend crested auklet chicks from ectoparasites, and this could be part of the parental care that adults provide. This may be analogous to the practice noted in other avian species of adding green plants to nests. Incorporation of plant volatile materials into avian nests may impair development of mites and bacteria (Clark and Mason, 1988; Clark, 1991), improve vigor of nestlings (Gwinner et al., 2000), and generally defend against ectoparasites, bacteria or fungi (Hart, 1997). Crested auklet hicks have only downy feathering during the first week (Jones, 1993a). Downy seabird chicks are more vulnerable to ticks in part because it is easier for the ticks to attach to skin (H. Douglas, pers. obs.). Increased chemical emissions could help to defend the chick from ticks, since octanal is an ectoparasite repellent (Douglas et al., 2004; 2005). Enhanced aldehyde emissions could also help interfere with the tick's chemoreception. A chick brooded under the wing of its adult would be close to the source of the odorant emissions. The

crested auklet odorant appears to function in a manner analogous to commercial repellents in that it overwhelms the ectoparasite's sensory apparatus (Douglas et al., 2005). The Yu'pik residents of St. Lawrence I., Alaska have noted that the citrus-like odor of crested auklets is particularly noticeable when the chicks hatch (W. Wongitillin, pers. comm.). This observation suggests that the seasonal patterns we detected could be indicative of a general seasonal pattern of odor emissions in crested auklets.

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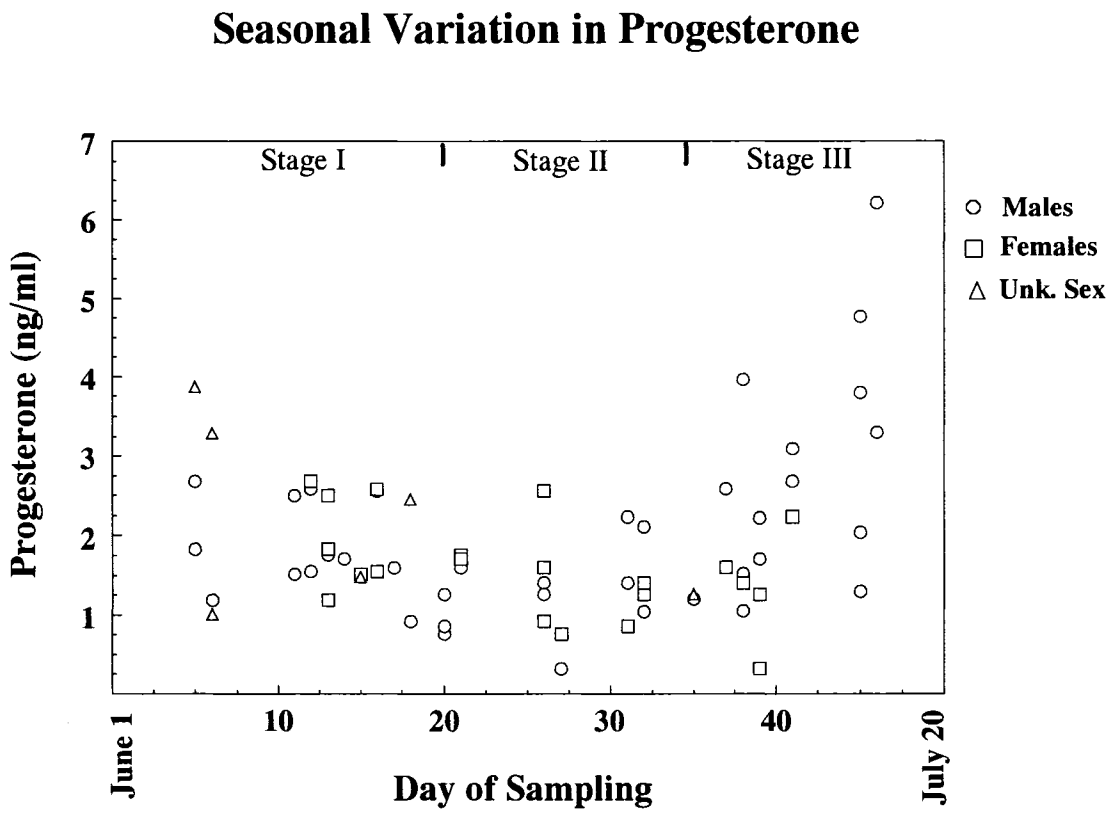

Figure 7.1. Seasonal variation in circulating levels of progesterone among adult crested auklets at Big Koniuji I., Alaska in 2002.

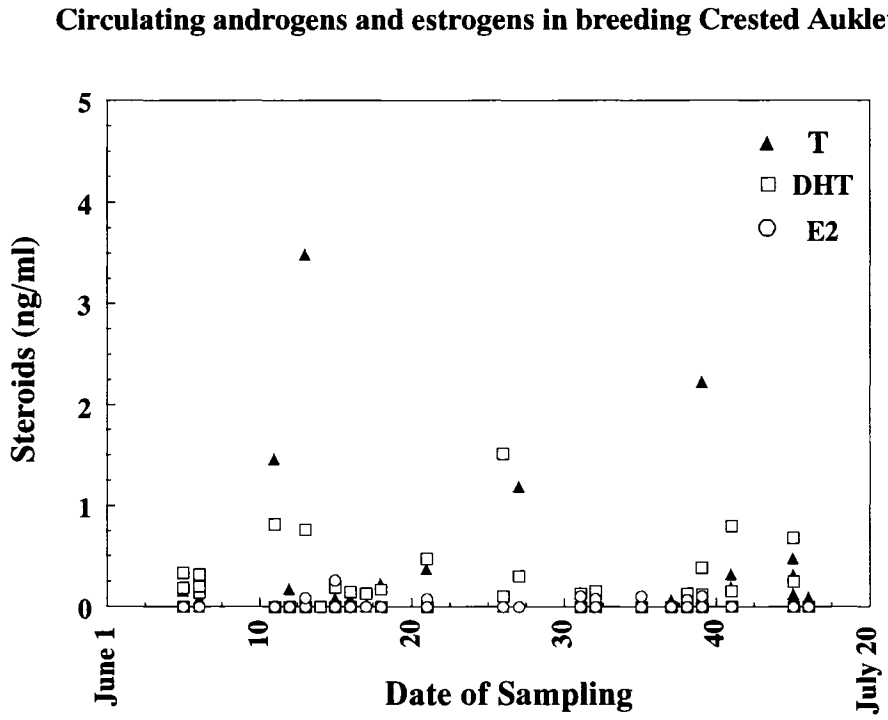

Figure 7.2. Seasonal patterns of androgens and estrogens in breeding adult crested auklets at Big Koniuji I., Alaska in 2002. Testosterone (T) was found in 42% of samples (0.05-3.49 ng/ml). Dihydrotestosterone (DHT) was found in 41% of samples (0.1-1.52 ng/ml). Estradiol (E_2) was found in 17% of samples (0.07-2.23 ng/ml).

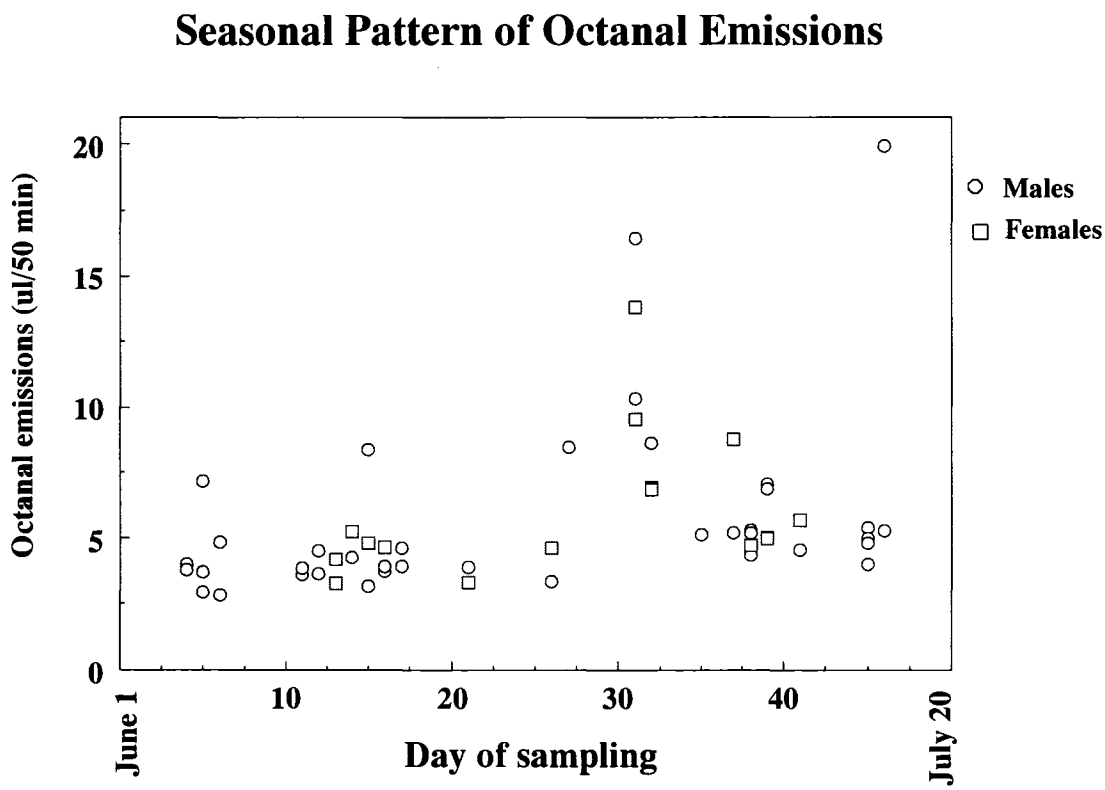

Figure 7.3. Seasonal pattern of octanal emissions in crested auklets at Big Koniuji I., Alaska in 2002. Values for breeding adult males are indicated by circles and values for adult females are indicated by squares.

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Chapter 8: Mutual anointment with chemical defense is a social and sexual ritual in crested auklets (*Aethia cristatella*)[1]

Abstract

Male and female crested auklets (*Aethia cristatella*) engage in mutual anointment with defensive compounds during courtship. Both sexes produce a citrus-like odor that is secreted into plumage from specialized wick feathers. Males display to females with a horizontal posture, outstretched neck and choking call. Females rub neck, head and bill over scent-laden plumage of males. Females then reciprocate with males in the same manner. The primary sources for odor constituents are specialized wick feathers, and these were located in the intrascapular region. High concentrations of aldehydes were found in wick feathers with the corresponding fatty acid methyl esters and carboxylic acids, suggesting that the odorant of crested auklets is synthesized from fatty acids. The concentrations of aldehydes measured in contour feathers were approximately two orders of magnitude less than that found in wick feathers. These concentrations were more than sufficient to interfere with tick locomotion in bioassay trials. The highest concentrations of aldehydes were found on the crown and nape, but no wick feathers were found there. This demonstrates that mutual anointment behavior serves to distribute aldehydes over the head and neck where the birds cannot self preen. High concentrations of aldehydes

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were recovered from accessory bill plates, demonstrating that the bill helps to distribute the odorant in plumage during mutual anointment behavior. Experiments with a naïve captive population suggest that behavioral response to the odorant peaks during early courtship. Higher concentrations of the synthetic odorant also elicited a stronger response from this captive population. Presentation of synthetic analogs of *Aethia* odorants in nature elicits exaggerated stereotyped display behaviors, including anointment behaviors. However, crested auklets responded to synthetic whiskered auklet odor as strongly as synthetic crested auklet odor.

Keywords: anointment, courtship, chemical defense, allopreening, ectoparasites, aldehydes, crested auklet, whiskered auklet

Introduction

Anointment behaviors are widespread in birds and mammals. Nonhuman primates are known to self-medicate with aromatic plant materials and arthropods and this behavior is hypothesized to serve as a defense against ectoparasites (reviewed in Weldon et al. 2003, Weldon 2004, Weldon and Carroll in press). Many species of birds anoint their plumage with plant and animal substances. This behavior was described as "anting" based upon the many observations of birds rubbing ants in their plumage and exposing their feathers to ant colonies (Simmons 1966). It has been suspected that these anointment behaviors could provide protection against ectoparasites, bacteria, and fungi (Simmons 1966, Ehrlich et al. 1986, Clayton and Vernon 1993, reviewed in Weldon and Carroll in press).

Vertebrates also produce their own endogenous secretions known or hypothesized to provide defensive benefits against ectoparasites and microbes (reviewed in Weldon and Carroll in press). In birds the best known examples are the pitohuis and *Ifrita kowaldi* of New Guinea (Dumbacher et al. 1992, 2000). These species may sequester a class of nerve toxins called batrachotoxins from dietary sources (Dumbacher et al. 2004). Some species have sufficient batrachotoxins in plumage to kill lice and deter predators (Dumbacher 1999), but the levels of the batrachotoxins vary seasonally and geographically, as well as among species (Dumbacher et al. 2000). Some pitohuis also produce a sour odor that may signal their toxic properties (Dumbacher et al. 1992), but the chemical identity of this odorant is not known (J. Dumbacher, pers. comm.). Preen oils secreted by the uropygial gland and preened into feathers by birds may also provide defensive benefits against lice, bacteria, or fungi (Moyer et al. 2003).

Mutual grooming in birds could reduce ectoparasite burdens (Brooke 1985), but mutual anointment with endogenous chemical defenses is unknown among birds. Crested auklets (*Aethia cristatella*) are planktivorous seabirds of Alaskan and Siberian waters that are highly social and nest in very large breeding colonies (Jones 1993a, Gaston and Jones 1998). Breeding adult males and females emit a citrus-like odorant during the breeding season that is noticeable at distances of up to 1 km from colonies and flocks at sea (Humphrey 1958, Jones 1993a, Douglas et al. 2001, Hagelin et al. 2003). The closely related whiskered auklet (*A. pygmaea*) also emits a citrus-like odorant, but the chemical composition differs between crested and whiskered auklets. For example, even-numbered aldehydes dominate the odorant of crested aukets, while odd-numbered

aldehydes dominate the odorant of whiskered auklets (Douglas et al. 2004). Behavioral experiments with a T-maze and lifelike taxidermic mounts suggested that the plumage odor of crested auklets has a general social function (Hagelin et al. 2003, Jones et al. 2004). Bioassays indicated the odorant could function as an ectoparasite repellent (Douglas et al. 2004, 2005b) although one study failed to demonstrate the efficacy of naturally occurring concentrations in plumage (Douglas et al. 2005a). During courtship crested auklets bury their bills in the nape feathers of prospective mates ("ruff-sniff"), and courtship concludes with an intertwining of necks (Jones 1993, Hunter and Jones 1999). The odor may play a role in courtship if the crested auklets could in some way assess the chemical odorant (Douglas et al. 2001, Hagelin et al. 2003, Jones et al. 2004). This study presents data regarding seasonal patterns of behavioral response to synthetic auklet odorant, discovery of specialized secretory structures, quantitative and descriptive measurements of chemical anointment, and a bioassay that tested effects of odorant constituents upon ticks.

Materials and Methods

Behavioral Studies

Captive behavioral trials - were conducted in a propagation facility at the Cincinnati Zoo and Botanical Gardens in 2003 and 2004 with taxidermic models and synthetic crested auklet odorant. The concrete enclosed facility measured 15 x 6.4 m, with a central freshwater pool measuring 7.5 x 3 m. The room housed 14 breeding adult crested auklets, ages 5-9 years in 2003 and ages 6-10 years in 2004. Two taxidermic

models were prepared from male crested auklet specimens of nearly identical size, morphology, and ornamentation. These specimens had been collected at St. Lawrence Island, AK in 2001 as part of another study (Douglas et al. 2005a). The specimens lacked a perceptible odor after the taxidermic procedures, and no constituents of the crested auklet odor were detected in methanol extracts of feather samples taken from taxidermic mounts. During behavioral trials the taxidermic mounts and odorant dispensers were rotated between opposite corners of the central pool. Synthetic analogues of the crested auklet's odorant, and an ethanol control were volatized from beneath these models. Synthetic constituents of the crested auklet's aldehyde odorant were measured in naturally occurring proportions (*n*-octanal 40%, *n*-hexanal 21%, Z-4-decenal 8%, *n*-decanal 3%, hexanoic acid 7%, octanoic acid 3%; Douglas et al. 2001a, 2004) with equal amounts of ethanol and each ingredient was added separately to dispensers. Two ingredients, Z-4 and Z-6 dodecenal, were not included in the experiment because these substances are not available commercially. In 2003 an odor gradient was created by adding 31.5 ml of odorant with 31.5 ml ethanol to a glass fiber wick (1" diam., MSI Wicks, Saratoga, IN). The control was an identical wick with 31.5 ml ethanol. These wicks were placed in open-ended steel pipes under rock piles. Models were placed on the rock piles directly above the wicks. In 2004 the active space of the chemical signal was reduced to close proximity of taxidermic models. In this year the same taxidermic models were placed on wooden platforms; holes drilled in the platforms permitted air movement from below. Wooden blocks holding red rubber serum stoppers (Fisher Scientific 03-215-5) were placed under the models. The experimental treatment

contained 3 ml crested auklet odorant and 3 ml ethanol. The control contained 3 ml ethanol. Odor dispensers and models were removed at the end of each trial. In 2003 the aldehyde odor was evident to human observers at approximately 6 m from the scented mount after one hour, but in 2004 the odor was only perceptible within 0.5 m of the model after one hour. Auklet behavior was observed from a small observation window. The following data were recorded: the number of crested auklets that approached each model, and the number that made behavioral displays to models during one-hour observation periods. Approaches were defined as the number of birds that walked to within 50 cm of the model's center point. This threshold was marked with white stones. Behavioral displays were described according to Jones (1993a) and Hunter and Jones (1999). Trials with taxidermic mounts were conducted weekly in 2003 and twice a week in 2004, from mid-May to early July. During 2004, experiments were also conducted with identical crested auklet decoys once a week. Identical crest ornaments of the same length and the same number of feathers were glued to the forehead of each decoy. Three working hypotheses were tested: 1) crested auklets respond preferentially to models scented with their odorant; 2) there is a seasonal pattern to behavioral response; 3) behavioral response is stronger when the chemical stimulus is stronger. Preferences were assessed by combining data for trials in each year and analyzing differences in the number of approaches and displays with McNemar's Test (Zar 1999). In 2004 all crested auklets were uniquely marked with colored leg bands, but not all auklets were individually marked in 2003. This was not a "blind" experimental design. Observers

knew which model emitted the scent in at least some of the experimental trials. This was due to the fact that not enough trained personnel were available on all dates.

Field experiments - were conducted with a "blind" design using the same models and decoys from captive experiments. The research was conducted from 25-30 June, 2005 at an auklet colony near Gambell on St. Lawrence I., AK (Lat 63° 47' N, Long 171° 45' W). These dates corresponded with pre-laying and possibly the onset of egg-laying as confirmed by observations of developing eggs in freshly collected specimens. In this experiment there were two treatments – synthetic analogues of crested and whiskered auklet scents, volatized from separate models. Taxidermic models and decoys were placed in platforms (8" x 8" x 2") cut from construction foam. The foam was painted to resemble a rock surface. Each odor constituent was volatized separately from an individual dispenser placed in a slot on the platform. The following chemicals and quantities were used for the crested auklet scent: 3.0 ml octanal, 1.5 ml hexanal, 0.5 ml decanal, and 0.5 ml Z-4-decenal (ratio 40:20:7:7). The whiskered auklet scent was 2.5 ml heptanal and 2.5 ml nonanal (ratio 1:1). All aldehydes were volatized from yellow rubber serum stoppers (Sigma-Aldrich) except the costly Z-4-decenal. This chemical was volatized from an 8 ml glass vial (Wheaton 225534). Each morning, models were placed on large rocks spaced at least 15 m apart. One model was placed per rock, and the models were moved to a different rock each day. Observations were made by H. Douglas with 10 x 40 binoculars from a natural hide well above the display rocks. The number of crested auklets that approached models each hour was recorded. Approaches were defined as birds that walked to within 40 cm of the model's center point, and this distance

was marked with four white stones. Behaviors were described in terms of frequency and duration.

Chemical analyses

Sample collection and preservation

Breeding adult crested auklets were captured at Sivuqaq Mountain near Gambell, St. Lawrence I., AK on June 23, 24, and 28. Samples were collected from an accessory bill plate on ten crested auklets to document the presence of odor constituents and determine concentrations. Sterile cotton-tipped swabs wetted with pure ethanol were wiped across the bill plate and then rinsed in a borosilicate glass vial containing 1.0 ml ethanol. The vials were sealed with a Teflon-lined cap and vapor seal (Duraseal stretch film). Seven breeding adults were sacrificed, and large samples of feathers were collected from two areas of the body – the mantle (specifically intrascapular) and the nape and crown. Each feather sample was placed in a borosilicate glass vial (Teflon-lined cap) containing 2.0 ml of methanol with dimethylhydrazine (3% by volume). Small samples of feathers were also collected from the mantle of 12 adult crested auklets in a captive propagation facility at the Cincinnati Zoo on May 12-13, 2004. Each of these feather samples was placed in a borosilicate glass vial (Teflon-lined cap) containing 1.0 ml of methanol with dimethylhydrazine (3% by volume). In each case, contour feathers were grasped with forceps and cut with scissors to avoid contamination with human skin oils. Vials were sealed with vapor seal to prevent volatization of solvent. After chemical analysis, feather samples were dried and weighed with an analytical balance (Mettler Toledo AG285).

Photoperiod in the zoo facility was maintained on a schedule similar to the eastern Aleutian Is. According to that phenology birds in the wild would have been emitting the odorant by the date that birds were sampled at the zoo. I have noticed that he citrus-like odor of crested auklets is conspicuously evident at the latitude of the Shumagin Is. by the second week of May (Big Koniuji I., AK 2002). However, the citrus-like odor of crested and whiskered auklets was not noted at the Cincinnati Zoo in 2004, nor in any year before or since that date.

Analytical procedures

Gas chromatography-mass spectrometry was carried out in the SIM mode (selective ion monitoring) using a HP5890 Series II Gas Chromatograph equipped with a 20 m x 0.25 mm, 5% phenyl siloxane column (Alltech), and a HP5972 Series Mass Selective Detector. The injector and detector temperatures were held at 250° C throughout, and the column flow was 1.0 ml/min. The instrument was programmed from 60 to 250° C in two stages. The first level increased at a rate of 4°C/min to a final temperature of 120° C and remained at that temperature for 4 min. The second level increased at a rate of 8° C/min to a final temperature of 250° C and remained at that temperature for 2 min. Mass spectra were obtained with an HP 5972 Series Mass Selective Detector. Retention time and ion abundances were obtained in EI mode from replicate standards (listed below) at different concentrations. The most abundant ions detected in EI mode were chosen for selective ion monitoring (SIM), and dwell times (milliseconds) were set for each ion according to its relative abundance. Subsequent analyses of standards in SIM mode showed that the selected parameters consistently

discriminated target analytes and obtained well-defined peaks at a range of concentrations.

Quality control was ensured by the inclusion of blanks, duplicates, internal standards, augmented standards, and calibration standards run at intervals in sequences at the frequency of 5-10% of total samples. Standards were prepared by serial dilutions in methanol and pure ethanol (see list of chemicals at end). Undecenal (97% Undecyclenic Aldehyde, ACROS Organics, C.A.S. 112-45-8) was used as an internal standard. Internal standard was added to all samples. Precise quantities of standards were measured with an Eppendorf Pipette (Model 4710). Accuracy and precision of the pipette was determined by replicate weighing of 10 µl samples of distilled water with a Mettler AE163 analytical balance ($x = 9.94 \times 10^{-3}$ g; S.E.= $\pm$ 8.38×10^{-5}; S.D. = 3.25×10^{-4}). Calibration curves were calculated from standards at three concentrations (8, 12 and 16 µl/ml) for each analysis, and the values obtained indicated high accuracy and precision (e.g., R^2=0.999 Hexanal; R^2=0.998 Octanal; R^2=0.994 Z-4-decenal; quantification contour feathers; R^2=1.000 Hexanal; R^2=0.999 Octanal; R^2=0.983 Z-4-decenal; R^2=0.999 Decanal, quantification wick feathers).

Chemistry and Morphology of Wick Feathers

Specialized feathers that release the auklet odorant to plumage were described for the first time in this study. I called them wick feathers because they have a morphological appearance and function similar to wicks. These wick feathers were dissected from frozen specimens and scaled on fine analytical balances (CAHN C-30 Microbalance and Mettler Toledo AG 135). After scaling, the wick feathers were

extracted in small volumes (20-50 μl) of pure ethanol. Analytical methods were similar to those used for feather extracts and bill swabs. Fatty acid methyl esters were evident in the first GC extractions of wick feathers, so standards for fatty acid methyl esters (FAMEs) were included in analysis of wick feathers. SIM parameters were adopted for FAME according to the same methodologies described above. GC injections for chemical analysis were performed with a handheld syringe because of the small volumes of tissue extractions. Internal standard was added to all samples. All uropygial feathers were clipped from 5 adult crested auklet specimens (3 males, 2 females; 4 specimens, St. Lawrence June 2005; 1 specimen, Little Diomede I. June 2004), extracted in methanol, and analyzed by the same methods for comparison with wick feathers.

Tick Bioassays

The effect of synthetic crested auklet odorant on tick activity levels was studied at low concentrations of crested auklet odorant. Filter paper (Whatman Qualitative 1, 15.0 cm) was trimmed to fit in Petri dishes (Falcon 1058, 150 x 15 mm). Graph paper with 4 mm grids was trimmed to exactly the same size and placed on top of the filter paper. A small hole was drilled in the lid of the petri dish and a 15 cm length of Teflon tubing (Bel-Art Products 062 PE tubing, Cat. No. 21852-0062) was fixed in the lid with tape. There were five paired dishes (experimental and control) for each treatment. Both experimental and control dishes had 1.8-2.0 ml solution (3 pts. distilled water:1 pt. glycerol:1 pt. ethanol) added to filter paper with a Hamilton syringe. Experimental treatments had 1% or 0.5% crested auklet odorant (40% octanal, 21% hexanal, 8% Z-4-decenal, 8% hexanoic acid, 3.5% decanal, 3.5% octanoic acid) in the solution. The filter

paper with its added solution was placed in the Petri dish and the graph paper was laid over top. Petri dishes were sealed around the edges with tape to hold them in place. Nymphs of laboratory-reared *Amblyomma americanum* were obtained from Oklahoma State University for this experiment. Each tick was used in one experiment, and then it was destroyed. A fine-haired camel brush was used to transfer the ticks into the Petri dishes, through the hole in the lid. Three nymphs were placed in each dish. One minute focal observations were timed with a stopwatch for each tick. Rate of locomotion was measured using grids; each grid was 4 x 4 mm. Replicate observations (8-9) were performed during the initial 5 hrs. After the first hour, carbon dioxide (a host cue) was supplied to each plate immediately preceding each observation by human exhalation for 5 sec through the Teflon tubing into the Petri dish. This ensured that all ticks were motivated to search for hosts during counts. All the observations for each dish were pooled, and the mean locomotion rates were compared using a paired t-test. Significance for the 0.05% treatment was tested with a one-tailed test. A one-tailed test is justified when the direction of the hypothesized effect is predictable (Zar 1999, p. 96). Estimates of the aldehyde exposures in bioassays were obtained by setting up Petri dishes in the exact manner of bioassays with the following treatments - 0.1, 0.5, 1.0, 2.0% crested auklet odorant. After one hour, three samples of three grids each (48 mm^2) were cut from each treatment. These samples were extracted in 1.0 ml methanol. Linear regressions were performed for results of hexanal, octanal, and z-4 decenal at four concentrations (0.1, 0.5, 1.0, 2.0 percent) in order to determine the average chemical exposure for the 1.0 and 0.5 percent bioassays.

The following chemicals were used for behavioral trials, chemical analysis, and bioassays: octanal 99% (C.A.S. 124-13-0), hexanal 96% (66-25-1), decanal 95% (11231-2), heptanal 95% (111-71-7), hexanoic acid 99+% (142-62-1), octanoic acid 99% (124-07-2) all by ACROS Organics; Z-4-decenal, 95% (21662-09-9) by Lancaster Synthesis, and nonanal, 97% (124-19-6) by Alfa Aesar. The following fatty acid methyl esters were used as standards: caproic acid methyl ester 99% (106-70-7), caprylic acid methyl ester 99.8% (111-11-5), capric acid methyl ester 99% (110-42-9), and lauric acid methyl ester (111-82-0), all by TCI America. Trace standards used in chemical analysis were 1-octanol 99% (111-87-5) by Avocado Research Chemicals Ltd., undecyclic aldehyde 97% (112-44-7) by ACROS Organics, and tridecanal 94% (10486-29-8). Methanol (67-56-1, HPLC grade, ACROS Organics) was used as the solvent for feather extractions and standards. The chemical agent used to derivatize aldehydes in feather extracts and standards was unsym-dimethylhydrazine, 99% (57-14-7, ACROS Organics). This was diluted to 3% in methanol since laboratory trials indicated this amount was sufficient to derivatize aldehyde concentrations in plumage. Pure ethanol (Quantum Chemical Corp., 200 proof dehydrated alcohol, U.S.P. Punctilious) was used as the solvent for bill swabs, for wick feather extractions, and for preparing chemical standards. It was also used as 20% of the solution in tick bioassays, along with 60% distilled water, and 20% glycerol (99+% purity, C.A.S. 56-81-5, Sigma-Aldrich). Aldehydes are hydrophobic and the addition of glycerol and ethanol were required to emulsify the solution.

Results

Behavior

Behavioral observations – Two types of behavior were observed in wild crested auklets that suggested mutual anointment. Putative mates pulled the side of their bill in a preening motion across the mantle feathers and up over the hindneck, nape, and crown of their partners. This behavior was observed in crevice entrances at Big Koniuji I., AK, at the extreme southeastern extent of the crested auklet's breeding range. It was also observed at St. Lawrence I. near the northwestern extent of the breeding range. A second behavior is part of the courtship ritual, and this was observed multiple times at St. Lawrence I. The male stretched out his head and neck in a nearly horizontal posture and made a vocalization and accompanying motion similar to the choking call of gulls (Groothius 1989). This behavior was also similar to a posture that birds adopt when they solicit allopreening (Verbeek et al. 1981). The female approached from the side and rubbed the underside of her neck over the male's nape and neck stroking towards the male's head. Then the female adopted the horizontal neck position. The male lifted his head and neck over the female's neck, rubbed the underside of his neck and breast over the top of her mantle, hindneck and nape. This was reciprocated several times between males and females and afterwards some pairs entered rock crevices together, presumably to inspect nest sites. Males and females were often distinguishable in paired displays at St. Lawrence I. Males have a thicker "bull" neck, and females had a more slender neck (H. Douglas, pers. obs.). Males and females are also distinguishable by their bill morphology (Jones 1993b). Mutual anointment in crested auklets was often followed

immediately by preening around the face and bill. Male advertisement displays sometimes attracted multiple birds that rushed in to form a "scrum" (a behavior described in Jones 1993a). These birds placed their bills in the mantle feathers of the displaying male. Approaches by females to males were more readily tolerated than approaches by males to males.

Captive Trials -- In captive behavioral trials, crested auklets responded more strongly to the scent of their own species than to the ethanol control. Furthermore, the strongest behavioral responses were during early courtship. This was shown in 2003 when 8 individuals approached the aldehyde-scented model on June 3, and 7 individuals approached the treatment on June 11 (Fig. 8.1). No auklets approached the ethanol scented model on these dates. Statistical interpretation across trials dates was limited because not all crested auklets were individually marked with colored leg bands; however, there were more approaches to the scented model than the control (19 vs. 7). The peak in behavioral displays occurred on June 3 (Fig. 8.2). Five crested auklets made displays towards the scented model on this date, but no crested auklets made displays to the control models on any date (5 of 14 vs. 0 of 14; $Z=2.24$, $P_{1\text{-tailed}}=0.01$, McNemar's Test). Displays occurred in sequences (>15 secs) and included some behaviors typical of advanced stages of courtship. The dates with the greatest number of responses corresponded to the early courtship phase in the crested auklet's breeding chronology.

In 2004, there were 14 approaches to the scented model and 6 approaches to the control. Most of the breeding adults (12 of 14) approached the aldehyde-scented model at least once during the course of the experiment while only 5 approached the control (Z=

-2.33, $P_{1\text{-tailed}} < .01$, McNemar's Test). The peak in approaches occurred on June 8 when 6 individuals approached the scented model (Fig. 8.1). This was also the peak in behavioral displays (3 birds; Fig. 8.2). Interestingly, the peak in approaches to decoys with the scented treatment occurred on the subsequent trial date, June 10. Three crested auklets approached the scented decoy on that date. No birds approached decoys on any other date. More displays were made to the aldehyde-scented taxidermic model than to the ethanol treatment (6 vs. 3), and more individuals made displays to the aldehyde-scented treatment than to the ethanol treatment. This difference was just significant (Z= -1.63, $P_{1\text{-tailed}}=0.05$, McNemar's Test). In comparison to 2003, the intensity of behavioral response was qualitatively less intense. Displays in 2004 were single behaviors of relatively brief duration (<10 sec) rather than the extended behavioral sequences observed in 2003. Birds that approached the aldehyde-scented treatment in 2004 were likely to approach the model again during a trial. When all of these approaches were counted, there were 24 approaches to auklet-scented models and 6 approaches to control models. The peak in behavioral response (early June) corresponded with early courtship. There was no preference for either taxidermic model. In 2004, 8 individuals approached taxidermic model #1, and 10 individuals approached model #2 (Z=0.32, $P_{1\text{-tailed}}=0.38$, McNemar's Test). In 2003, 11 birds approached model #1 and 7 birds approached model #2 (position of models was not recorded for one trial).

Field Trials – Crested auklets did not prefer the synthetic odor of conspecifics to that of whiskered auklets. The number of approaches to crested auklet and whiskered auklet treatments was nearly equivalent (55 v. 64), respectively, and there was no

difference in the hourly rate of approach (3.05 v. 3.56, $t_{2\text{-tailed}(.05)}$= .38, p=.71). More crested auklets approached the decoys scented with the crested auklet scent than the whiskered auklet scent (10 vs. 2); however, when the hourly rate of approach was compared the difference was not statistically significant (0.59 vs. 0.12, $t_{2\text{-tailed}(.05)}$=1.74, p=.10). The duration of approach was similar to taxidermic models with whiskered auklet and crested auklet scents (65 vs. 43 s; $t_{2\text{-tailed}(.05)}$=1.87, p=.06). There was no preference for either taxidermic model; the rate of approach and the duration of approach did not differ for either taxidermic mount (p=.31 and .57, respectively). There were sequences of behaviors that suggested crested auklets were strongly attracted to both whiskered and crested auklet scents. Sniffing was characterized by a low sweeping motion of the head and neck, back and forth while walking over the landing rock, as though attempting to locate the source of the odor. Displaced anointment behavior involved rubbing and dragging the bill on the foam platform and the rock surface near the foam. This behavior could also be termed "displaced ruff-sniff" because it is very similar in its motion to the ruff-sniff behavior observed in courtship. This displaced anointment behavior was interspersed with sniffing as crested auklets honed in on the source of the odor. Crested auklets that located the odor dispensers (sunken in the foam platforms) placed their bills directly over the dispensers. Some tilted their head as though bringing their nostrils close to the dispensers, and others tapped the dispensers with their bills. Subsequently some crested auklets climbed onto the platform or climbed onto rocks holding down the platform, and they reached over to touch their bills to the hindneck and mantle feathers of the models. Four crested auklets did this to models associated with the

crested auklet scent, and five made contact with a taxidermic model associated with the whiskered auklet scent. In all these behaviors, the response to the whiskered auklet scent was at least as strong as the response to the crested auklet scent. For example, sniffing behavior was noted in 12 individuals responding to the whiskered auklet scent and in 4 individuals responding to the crested auklet scent. Anointing behavior was the most dramatic. This occurred four times when crested auklets climbed onto the platform holding the whiskered auklet scent. In each case the male or female rubbed its body vigorously on the male taxidermic mount. In one case a male crested auklet climbed onto the back of the model and rubbed itself on the intrascapulars and hindneck while intertwining its neck with the model's neck on one side and then the other. In another case a crested auklet made contact with the model 17 times, preening and rubbing on the intrascapulars, hindneck, and nape during 1 min. 52 s. The duration, frequency, and intensity of these behaviors were greater than was observed in individual bouts among displaying pairs. Even so, the male self-advertisement displays were subdued compared to what is most commonly observed in nature. Males that were engaged in sniffing and displacement preening gave soft trumpeting calls in a hunched posture. Typically the trumpeting call is a loud vocalization, and the display is given in an erect posture with the bill pointing skyward (Jones 1993a, Hunter and Jones 1999). Overall, behavioral responses appeared to be focused on locating and contacting the odorant; birds attempted to contact the chemicals with the bill; and some of the behavioral responses to the synthetic odorant were prolonged and intense.

Chemical Analysis

Contour feathers – Previously reported odor constituents (hexanal, octanal, decanal, Z-4-decenal, dodecenals, Douglas et al., 2001, 2004) were detected in all feather samples. Putative oxidation products (hexanoic and octanoic acid) were not detected in all samples. Discrete peaks for the two dodecenal isomers (Z-4 and Z-6) were sufficiently distinct in three individuals to permit calculation of peak areas, and this showed that the two isomers occurred in a 1:1 ratio (consistent with Douglas et al., 2004). Chemical concentrations were higher in the plumage of the crown and nape than in the mantle feathers (hexanal= 458 v. 119; octanal= 490 v. 96; Z-4-decenal= 344 v. 65, μg/g).

There were consistent interindividual differences in aldehyde concentrations of crested auklets. The highest concentrations of hexanal and Z-4-decenal on the crown and nape co-occurred in the same individuals ($r=.995$, $p<0.001$). The same was true for hexanal and Z-4-decenal in the mantle feathers ($r = .83$, $p=.04$), and hexanal and octanal in the mantle feathers ($r_s= .90$, $p=.037$). Individuals with the highest concentrations of octanal in crown and nape plumage also had the highest concentrations of Z-4-decenal in mantle feathers ($r=.95$, $p=.01$). These data help to show that individual crested auklets differ in the amounts of chemical odorant they emit, as was also shown by another method in Chapter 5.

Trace constituents reported for crested auklets (octanol, nonanal, undecanal, tridecanal; Hagelin et al., 2003) were not detected in contour feathers, with the exception of heptanal. Heptanal was detected at trace amounts in the mantle feathers of one individual but not on feathers of nape and crown.

Feather samples collected from crested auklets at the Cincinnati Zoo did not have detectable concentrations of odor constituents in feather samples. There was barely a trace in 3 samples that occurred at the retention time for decanal dimethylhydrazone. No evidence of other odor constituents was found in any of the 12 samples.

Concentrations on bill - Chemical constituents of the crested auklet odorant were detected in swab samples from the lower mandible of ten crested auklets; however, not all compounds were detected on mandibles of all individuals (hexanal=3.8 ± 0.6, n=7; octanal=6.8 ± 0.38, n=10; Z-4-decenal=6.9 ± 2.3, n=7; decanal=8.2 ± 4.3, n=6; dodecenal=4.3, n=1; $\mu g/cm^2$). The highest concentrations were 6.0 ($\mu g/cm^2$) for hexanal; 9.5 for octanal; 20.6 for Z-4-decenal; and 29.5 for decanal ($\mu g/cm^2$).

Tick Bioassay

Ticks placed on grid paper above the 1% crested auklet (CRAU) odor treatment moved more slowly than controls (10.8 ± 1.6 vs. 27.6 ± 1.6 grids), and this difference was highly significant (paired samples $t_{(0.05)2\text{-tailed}}$= -15.1, p<0.001, df=14). Ticks in the control treatment moved at an average rate of 110 mm/min, and ticks exposed to the 1% odor treatment moved at an average rate of 42 mm/min. Ticks exposed to the 0.5% CRAU treatment were not as strongly affected, but the difference was significant for a one-tailed t-test (p=0.046).

Chemical analysis showed that concentrations of aldehydes in bioassays were less than or equal to concentrations found in crested auklet plumage. The concentrations of aldehydes in graph paper for the 1% bioassay were 359 μg/g octanal, 251 μg/g hexanal, and 194 μg/g Z-4-decenal. These measurements were obtained from GC/MS analysis of

graph paper. These values were similar to the calculated amounts added to the filter paper (e.g., 354-389 μg/g octanal).

Morphology and chemistry of wick feathers

Wick feathers are translucent hair-like feathers rooted in small globular tissues that have a creamy orange color (Fig. 8.3). Scanning electron microscopy suggested that the wick feathers may have a hollow or at least a porous core (Fig. 8.4). Aldehydes may be conducted up through the wick core. The average mass of wick feathers was 1.1 mg (S.E. = 0.88, n = 25), and the globular tissues at the base of the wick were approximately 0.5 mm in diameter. All wick feathers were found in the intrascapular region; no wick feathers were found in the nape or crown of the specimens. Chemical concentrations were analyzed for 25 samples of wick feathers. These samples were dissected from the intrascapular region of seven frozen specimens of crested auklets taken at St. Lawrence I. and Little Diomede I., AK. Two samples contained 5 wick feathers, one sample contained 4 wick feathers, one sample contained 2 wick feathers, and 21 samples contained 1 wick feather. Major odor constituents previously reported from crested auklets (Douglas et al., 2001a; Hagelin et al., 2003; Douglas et al., 2004) were found in 22 samples, and fatty acid methyl esters were found in 16 samples. Fatty acid methyl esters are a class of compounds not previously reported for the crested auklet's odorant. The average concentration of total aldehydes was 109.8 mg/g (S.E. = 50.6, n = 22), and the average concentration of total FAME was 28.7 mg/g (S.E. = 18.6, n=16).

All of the major aldehyde constituents of the crested auklet odorant were detected in wick feathers. These constituents are even-numbered aldehydes 6-12 carbons in length

(hexanal, octanal, decanal, Z-4-decenal, Z-4-dodecenal, Z-6-dodecenal; Douglas et al. 2001a, 2004). Not all of these compounds were found in the same wick feathers. Hexanal and octanal co-occurred in 15 samples (27.9 ± 13.9 mg/g and 33.2 ± 15.5 mg/g, respectively), and hexanal was detected at trace amounts in one additional sample. Decanal (24.6 ± 11.3 mg/g, n = 18) and Z-4-decenal (24.2 ± 11.5 mg/g, n=20) co-occurred with hexanal and octanal in 14 samples and also co-occurred in 4 additional samples. Dodecenals were found in 20 samples (49.7 ± 19.4 mg/g). Decanoic and dodecanoic FAME were not previously reported for the crested auklet odorant. These compounds occurred in 8 and 20 samples, respectively. Decanoic FAME averaged 35.3 ± 30.4 mg/g, and dodecanoic FAME averaged 12.8 ± 9.1 mg/g. Octanoic FAME occurred in 3 samples (0.92 ug/g to 213.0 mg/g) and was detected at trace amounts in one additional sample. Hexanoic FAME did not occur in any wick feathers, but it was found in the follicle tissues surrounding one wick feather. Hexanoic acid and octanoic acid were found in 10 and 12 samples, respectively. Calibration curves for these carboxylic acids were not robust, but approximate concentrations ranged from 56 μg/g to 305 mg/g. Heptanal, octanol, and nonanal were detected at trace amounts in 3, 2, and 2 samples, respectively. Undecanal and tridecanal were not detected. Major odor constituents (octanal, hexanal, decanal, Z-4-decenal, dodecenals) were not detected in tissues adjacent to the wick feathers, but hexanoic and octanoic acids were detected in these adjacent tissues. No major odor constituents were found on the surface of skin that had been plucked of feathers (as determined by scotch tape application to skin and methanol

extraction). This indicated that the aldehyde odorant constituents (with the exception of decanal) were only released from wick feathers.

Decanal (16.4 ± 6.4 μg/g) and decanoic FAME (16.9 ± 5.4 μg/g) were detected in the extracts from uropygial feathers of 5 crested auklet specimens (3 males, 2 females) taken at St. Lawrence I. and Little Diomede I. Decanal is the only odorant constituent that has been reported at significant levels in both crested auklets and whiskered auklets. No other odor constituents were detected in uropygial feathers.

Discussion

Mutual anointment with defensive chemicals is a social and sexual ritual in crested auklets. During courtship male crested auklets solicit behaviors from females that results in the distribution of defensive chemicals in plumage. Males and females rub their bills, faces, necks and breasts over wick feathers that contain high concentrations of aldehydes and secrete these into plumage. Ectoparasites that are directly exposed to secretions of wick feathers could be killed or paralyzed. Average concentrations of aldehydes in plumage are less than wick feathers by approximately two orders of magnitude, but as shown in this study these concentrations are sufficient to impair ticks. Average concentrations of aldehydes on an accessory bill plate were two orders of magnitude greater than those used in a bioassay that was strongly repellent to mosquitoes (e.g., octanal 0.048-0.052 μg/cm^2, 1% treatment, Douglas et al. 2005b). Concentrations of aldehydes near the face may also be strongly repellent to ectoparasites. Crested auklets rub their faces in the same areas where they rub their bills, and chemical

repellents provide protection up to 4 cm from the anointed surface. Bare skin around the eyes and face are the areas most vulnerable to tick parasitism on birds (Reed et al. 2003). Previous studies have shown that constituents of the crested auklet odor kill, paralyze, repel, and impair ectoparasites in a dose-dependent fashion (Douglas et al. 2004, 2005b). The higher average concentrations of aldehydes on the crown and nape show that the mutual anointment behaviors help to distribute aldehydes over body regions where crested auklets are not able to preen themselves. This may help defend crested auklets against lice. Aspects of mutual anointment behavior recall allopreening. Crested auklets appear to preen display partners around the face and bill immediately after mutual anointment behaviors. These preening behaviors may remove lice that have become impaired by fumigation during mutual anointment.

The aldehydes found in the crested auklet's odorant are likely products of fatty acid biosynthesis. The only sources for most aldehydes (hexanal, octanal, Z-4-decenal, dodecenals) were specialized wick feathers located in the intrascapular region. These structures contained aldehydes with corresponding fatty acid methyl esters and carboxylic acids. Aldehydes were not found in tissues adjacent to wick feathers or on the surface of the skin. The uropygial gland is also a source of chemical secretions that are applied to plumage (Jacob and Ziswiler 1982); however, in this study only decanal (a minor odor constituent) was found in uropygial feathers. Therefore, the uropygial gland is probably not a source for most of the aldehydes. Wick feathers in crested auklets may be similar to fat quills found in Columbiformes, the resinous feathers of the Heart-spotted Woodpecker (*Hemicircus canente*), or the powder feathers of herons and egrets (Menon 1984). The

wick feathers might be similar to filoplumes or bristles (Stettenheim 2000), but they are not consistent with all characteristics of those feather types. For example, unlike filoplumes, the wick feathers do not always occur adjacent to other feathers, and some wick feathers are bi-lobed.

Aldehydes elicit behavioral responses from crested auklets. In behavioral trials conducted at St. Lawrence I., crested auklets appeared to search for odor dispensers with a conspicuous sniffing and searching behavior. This behavior was consistent with orientation by olfaction. The auklet colony on the mountain near Gambell is located in a windy environment, and the wind turbulence would have caused odor plumes of the volatile aldehydes to swirl over the rock surface. During sniffing crested auklets appeared to narrow their olfactory search as they honed in on the foam platform that emitted the aldehydes. Crested auklets rubbed their bills on the platform and the nearby rock surface. This motion with the bill was very similar to how the bill is used in mutual anointment behaviors with partners. Thus, it can be regarded as a displaced anointment behavior. It was also very similar to the way the bill is used in the "ruff-sniff" behavior that occurs during courtship. Some individuals also tapped the odor dispensers with their bills. These sequences of behaviors showed that crested auklets were attempting to locate and make contact with aldehydes in a manner analogous to mutual anointment behaviors. The presence of volatile aldehydes probably helps to promote anointment behaviors. Some crested auklets that engaged in sniffing, searching, and displaced anointment behaviors, subsequently made prolonged contact with taxidermic models. These birds rubbed themselves on the intrascapular region of taxidermic models where the aldehyde-

secreting wick feathers are located. This response was even more intense and of greater duration than what is observed between displaying pairs and for that matter in another field experiment with taxidermic mounts (Jones et al. 2004). This strong behavioral response was probably due to the presence of a strong aldehyde scent around models.

The crested auklet's citrus-like odorant may help mediate reproductive behavior, similar to pheromones and odors in other vertebrates (Agosta 1992, Wyatt 2003). In experiments with captive crested auklets, behavioral responses to chemical analogues of this odorant attained the highest frequency during early courtship. Furthermore, behavioral responses were greater in the year when the chemical odorant was stronger. Captive birds at the Cincinnati Zoo do not emit the citrus-like odor, and the reason for this is not known. These zoo birds were taken into captive care at age 5 days, and therefore they were naïve to adult social and sexual contexts of their species' citrus-like odor prior to this research. Clearly crested auklets are attracted to the citrus-like scent. This was also shown in pilot studies conducted with paired and identical artificial models. Crested auklets closely approached photographic models in 2 out of 3 trials at the Cincinnati Zoo, when the citrus odor was present with ethanol ($n \geq 5$ individuals and 7 approaches; Douglas et al., 2001b). No crested auklets approached the control photo model that emitted only ethanol. The importance of the odorant in reproductive behavior is also suggested by the fact that chemical emissions are correlated with progesterone in breeding adults (Chapter 7).

Wild crested auklets did not prefer the synthetic odor of conspecifics to the synthetic odor of whiskered auklets. The hourly rate of approach and the proximity of

approach did not differ statistically for taxidermic mounts and decoys that emitted crested and whiskered auklet scents. Whiskered auklets do not occur at St. Lawrence I., and the nearest breeding colonies for this species are far to the south in the Aleutian Is. (Byrd and Williams 1993).

The *Aethia* odorant could be a shared ancestral trait. Decanal is a common odor constituent of whiskered and crested auklets (Douglas et al. 2004). The biochemical pathways that produce the plumage odorants may have diverged in these species. Even so crested auklets exhibited the same characteristic responses to synthetic replicas of whiskered and crested auklet odor, and the scent of whiskered auklets was at least as attractive as the scent of crested auklets. The stimuli that were presented in field trials were stronger than what is emitted by a single bird in nature, and the responses were also of greater duration and higher intensity. This shows that sexual displays and anointment behavior can be motivated by aldehyde constituents of the crested auklet odorant, and the intensity of the signal may be as important for eliciting a response as the precise ratios of odor constituents.

Aldehyde odorants of *Aethia* auklets may have evolved as a chemical defense against ectoparasites and subsequently been assimilated as a chemical signal of mate quality (Douglas et al., 2001a). Birds that secrete higher quantities of the odorant may be more desirable mates for reasons predicted by current models of parasite mediated sexual selection (Møller et al. 1999). According to these models, parasite free mates are likely to provide better parental care and reduce the likelihood of transmitting parasites to mates and offspring. Also mutual anointment with a mate that secretes more chemical defenses

is likely to confer greater chemical concentrations to plumage and thus provide greater protection against ectoparasites such as ticks and lice. This is particularly true since there appeared to be a threshold effect on ticks at the average concentrations of aldehydes found in mantle feathers.

Sexual selection operating in ancestral populations of *Aethia* auklets may have led to a divergence in chemical signals. As has been shown in this study, new scents that suddenly arise to a higher intensity in a population can be as attractive as familiar odors, and this could have been the evolutionary basis for the interspecific chemical differences in the odorants of crested and whiskered auklets (Douglas et al., 2004).

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Lawrence I., Alaska was granted by Sivuqaq Native Corporation, Inc. Specimens were obtained at Little Diomede I. by G. Sheffield with permission of Native Village of Diomede. This research complied with all applicable U. S. laws and ethical guidelines pertaining to treatment of animals in behavioral research.

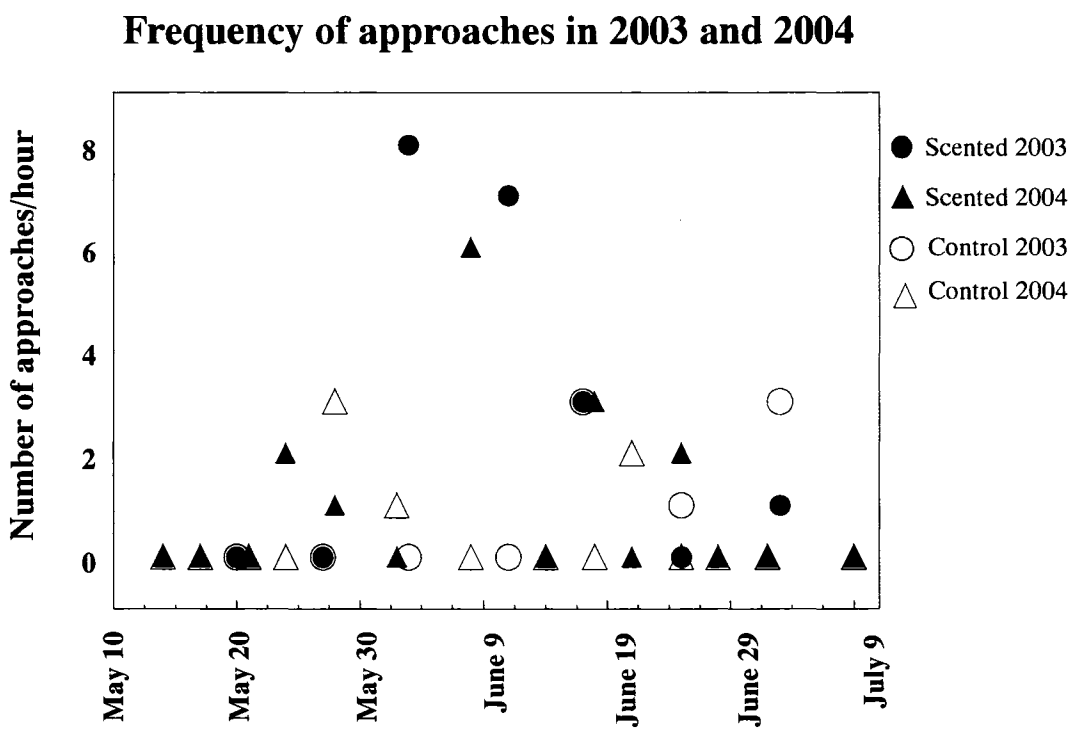

Figure 8.1. Captive crested auklets at Cincinnati Zoo approached taxidermic models with synthetic crested auklet odorant at a higher frequency than ethanol controls. A strong chemical signal was used in weekly trials during 2003, and a faint chemical signal was used in trials that were conducted twice a week in 2004.

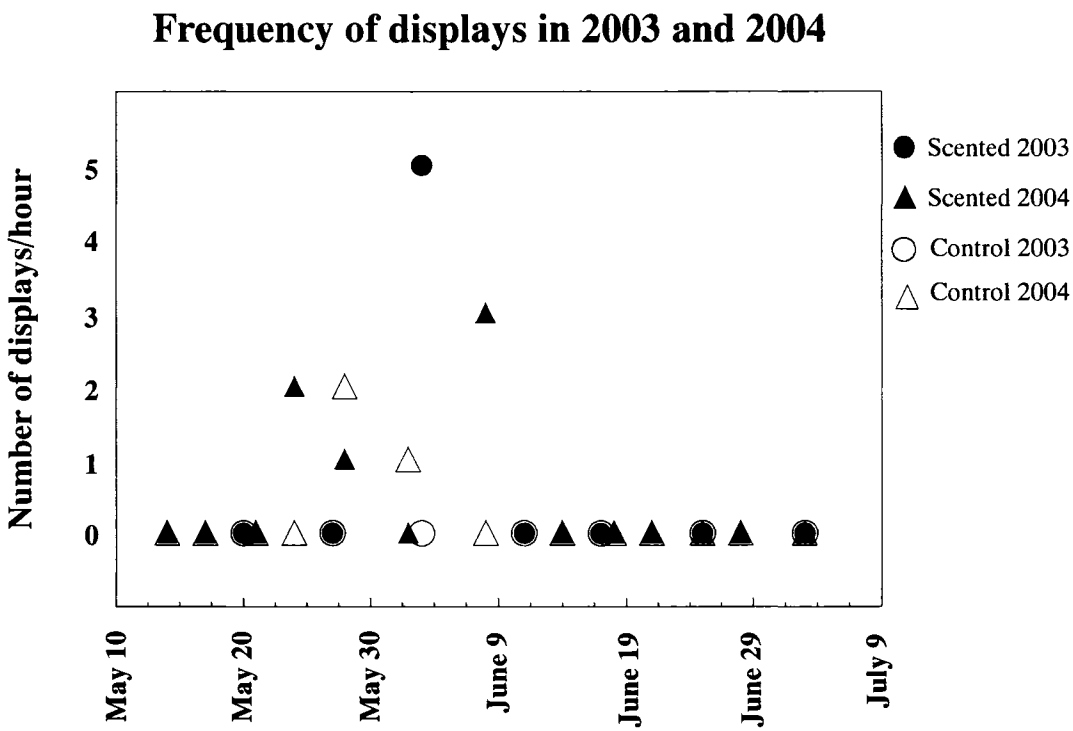

Figure 8.2. Captive crested auklets at Cincinnati Zoo displayed to models with synthetic crested auklet odorant at a higher frequency than controls. A strong chemical signal was used in weekly trials during 2003, and a faint chemical signal was used in trials that were conducted twice a week in 2004.

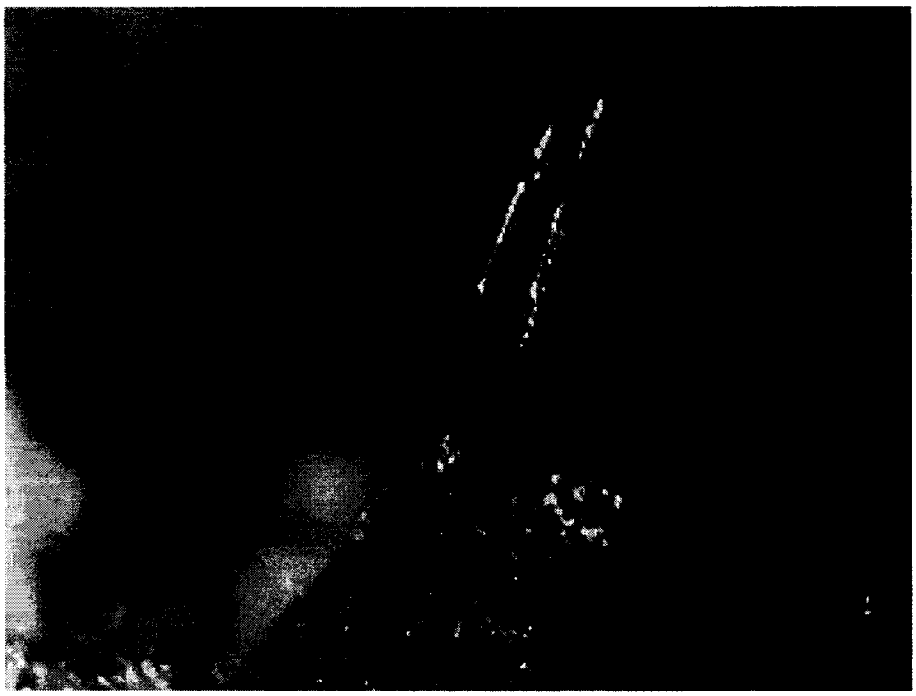

Figure 8.3. Image of bi-lobed wick feather from crested auklet specimen captured with video microscope. Wick feathers apparently release the aldehyde odorant to plumage. The fleshy tissue at the base of the wick contains high concentrations of odor constituents.

Figure 8.4. Electron microscope image shows a wick feather in cross-section. The wick appears to have a hollow or porous core (scale bar reads 25 μm). Image was obtained with an ElectroScan E2020 Environmental Scanning Electron Microscope (Advanced Instrumentation Lab, University of Alaska Fairbanks, courtesy Ken Severin).

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Conclusions

Ornaments and odors can serve as indicators of individual quality in crested auklets (*Aethia cristatella*). Evidence accumulated in this research showed that ornaments and odors do correlate with other indicators of quality. Crested auklets differ in the size of their crest ornaments, and crest size is negatively correlated with corticosterone in males (Chapter 6). Males with longer crest ornaments had lower baseline corticosterone levels and relatively low response in stress profiles. The opposite tended to be true for males with shorter crests. This suggests that males with longer crests cope better with cumulative physiological stress (Chapter 6). Crested auklets also differ in the amount of odor that they emit (Chapter 5). The average concentrations of aldehdyes in crested auklet plumage are sufficient to interfere with tick locomotion (Chapter 8). Interestingly, crested auklets had a very low incidence of tick parasitism, but one male with very low octanal concentrations was heavily parasitized by ticks (Chapter 5). The crested auklet odorant appears to have broad spectrum efficacy against ectoparasites. Mosquitoes were repelled by very low doses of synthetic odorant, and the results of mosquito bioassay trials were comparable to results obtained with commercial mosquito repellents (Chapter 4, Douglas et al. 2005b). Concentrations of odorant on the bill of crested auklets were much higher than concentrations used in low dose trials with mosquitoes (Chapter 8). Naturally occurring concentrations of aldehydes in tissues are sufficient to kill or paralyze lice (H. Douglas, unpubl. data). Therefore, it is reasonable to conclude that naturally occurring concentrations of aldehydes in crested auklet tissues and plumage are sufficient to interfere with parasitism by ectoparasites.

Crested auklets assess ornaments and odors when choosing mates. Previous studies have shown that length of the crest ornament in crested auklets is associated with sexual attractiveness (Jones and Hunter 1993) and dominance (Jones and Hunter 1999). Males and females display preferentially to lifelike models of the opposite sex when those models have longer crest ornaments (Jones and Hunter 1993, Jones and Hunter 1999). Males that have long crests tend to be paired with females that have long crests (Jones and Hunter 1999). Females with short crests are more likely to divorce and seek males with longer crests in a subsequent breeding season (Fraser et al. 2004). Behavioral experiments showed that crested auklets respond to synthetic analogues of their odorant during the courtship phase of breeding, and stronger scents elicit a higher frequency and higher intensity of response (Chapter 8). Behavioral observations also indicate that odor assessment and mutual anointment are part of courtship (Chapter 8). Therefore, it is logical to conclude that crested auklets assess odor when selecting mates.

Crested auklets could increase their fitness by selecting mates with larger crests and higher chemical potency. According to this study, males with larger crests are likely to have lower baseline levels of corticosterone and lower adrenocortical response to acute stress. Lower corticosterone and lower stress response suggest better physiological condition. Other studies have shown that corticosterone levels correlate negatively with reproductive success and survival in vertebrates (Lanctot et al. 2003, Love et al. 2004, Romero and Wikelski 2001). The crested auklet's citrus-like odorant interferes with the ability of ectoparasites to parasitize hosts. The efficacy of the odorant is dose-dependent (Chapters 2, 4, 8). Crested auklets that emit more odorant are likely to have greater

success at repelling ticks. Crested auklets may preferentially select mates with higher chemical potency. This would confer greater protection against ectoparasites for the mate and offspring, and protection against ectoparasites could favorably influence fitness.

The crest ornament is a conventional signal that costs little to produce and maintain (Jones et al. 2000). Social costs are likely incurred by the bearer in proportion to the degree of ornament expression, and those costs may be manifested as physiological stress (Chapter 6). These results support the claim that conventional signals incur a cost due to probing assessment by receivers, and in this case there is the likelihood of retaliation (Vehrencamp 2000). Higher signal expression in crested auklet males with larger crests may draw more challenges from strong, aggressive opponents (Maynard Smith 1982). Increased fighting could inflict physiological costs that reduce the quality of parental care (Chapter 6), and physical injuries that could imperil an individual's survival.

The crested auklet's odorant is similar to assessment signals in some respects. In order to assess the chemical signal, the receiver must expend time and increase its vulnerability to attack by the sender. Time spent assessing the chemical signal also increases the risk to predation. Even copulation attempts among auklets at sea can be terminated by predation of one partner by gulls (Hunter and Jones 1999). The sender adopts these same costs in deploying the chemical signal and also diverts metabolic products to production of the signal. Properties of the chemical signal may provide reliable information about condition of the sender. The quantity of octanal emission was

correlated with progesterone in male crested auklets (Chapter 7). It may also be negatively correlated with ectoparasite load (Chapter 5).

The aldehyde odorant emitted by crested auklets conformed to certain characteristics of chemical defenses described by Berenbaum (1995). Defensive compounds are usually secondary compounds which are derived from primary metabolites. These may be synthesized *de novo* or sequestered directly from food sources. Aldehydes, the major constituents of the crested auklet odorant, are secondary compounds that are probably derived from primary metabolites utilized in lipid metabolism. Chemical defenses are usually compartmentalized within specialized tissues to avoid self-poisoning, and there is usually a system for external delivery. In crested auklets the aldehydes that comprise the crested auklet's odorant are concentrated in specialized secretory tissues that I have called wick feathers. These wick feathers appear to release the odorant directly into feathers (Chapter 8). An optimal chemical defense should maximize the benefits of protection against natural enemies, but minimize the costs to the organism that deploys the chemical defense. This also appears to be the case with crested auklets. The concentrations of aldehydes in plumage are sufficient to interfere with ectoparasites and probably gain some fitness advantage. Chronic exposure to high levels of aldehydes could result in costs to crested auklets because aldehydes are harmful to various tissues (James 2000, Smiesko and Benfenati 2004, Soo 2002).

Some data for lice provided contradictory evidence regarding the efficacy of the crested auklet's odorant as a chemical defense. Brief exposures to naturally occurring concentrations of the crested auklet odorant in dermal tissues were sufficient to kill or

paralyze auklet lice (H. Douglas, unpubl. data). Also auklet lice were killed, paralyzed and otherwise impaired by very low doses of auklet odor constituents (Chapter 2, Douglas et al. 2004, H. Douglas, unpubl. data). Nevertheless, survival experiments showed that pigeon lice survived as well over treatments with crested auklet feathers and crested auklet specimens as they did over control treatments (Chapter 3, Douglas et al. 2005a). Another finding in Chapter 3 that contradicted general conclusions was that crested auklets had significantly higher ectoparasite loads than least auklets, even after adjusting for differences in host body size. Dale Clayton and I had proposed that crested auklets should have lower ectoparasite loads because they have a putative chemical defense that least auklets do not have. Instead, we found the opposite relationship. This combination of negative results suggested that aldehyde concentrations in feathers are not sufficient to kill pigeon lice and therefore might not be sufficient to kill auklet lice (Chapter 3, Douglas et al. 2005a).

Nevertheless, based upon additional research, it now appears that aldehyde concentrations in crested auklet plumage should be sufficient to have deleterious effects upon lice. The combined concentrations of hexanal, octanal and Z-4 decenal were approximately 1.29 mg/g in head plumage and 280 μg/g in mantle feathers (Chapter 8). These concentrations were greater or equal to the concentrations of aldehydes (217.4 ± 18.48 μg/g) used in the louse mortality trials reported in Chapter 2. Louse in those bioassays became moribund in less than 4 sec, and the aldehyde vapor rising through the feathers was sufficient to bring this about. Auklet lice that were exposed to nicks in the skin of a freshly killed crested auklet were instantly killed or paralyzed when aldehydes

were present in the tissues (H. Douglas, unpub. data). The crested auklet's odorant may cause a range of deleterious effects to lice depending upon the dose and duration of exposure. The lice could be paralyzed or impaired when aldehydes are released into plumage and this could augment the auklets' behavioral defenses against lice. Preening is the chief behavioral defense that birds use against lice (Cotgreave and Clayton 1994, Clayton et al. 2005). Birds move their beaks very rapidly during preening to damage lice in various ways. Clayton et al. (2005) recorded louse preening by pigeons with high speed video and determined that movements of the mandibles occurred at a rate as high as 31 times/second. This preening action damaged lice by decapitation, removing appendages, and lacerating the exoskeleton (Clayton et al. 2005). Chewing lice are specially adapted for locomotion on the feathers of their specific hosts (Marshall 1981, Bush et al. 2006). Lice that are impaired by fumigation from aldehydes would be more vulnerable to damage by preening because they are not able to move their appendages as rapidly to locomote through feathers.

Several factors could have influenced survival experiments reported in Chapter 3. Lice were segregated from plumage, and the efficacy of repellents diminishes with distance (Maibach et al. 1974). The experiments were conducted after the release of the aldehydes to plumage. Aldehydes are highly volatile and reactive and are unlikely to persist for a long time in plumage. Exposure to volatiles from plucked feathers or freshly killed specimens may not accurately simulate the exposures that occur in nature when crested auklets engage in mutual anointment. Another factor to consider is the larger body size of pigeon lice and the greater resistance against auklet volatiles that larger body

size appears to provide. My experiments at the University of Utah showed that pigeon lice were more tolerant of synthetic auklet aldehyde constituents than were auklet lice. Auklet lice (*Quadraceps, Saemundssonia, Austromenopon*) exhibited a progressive loss of motor control (sluggishness, immobility, spasmodic twitching, paralysis) at lower doses of auklet aldehyde than was true for pigeon lice (*Columbicola, Campanulotes*; H. Douglas, unpubl. data).

Louse populations on crested auklets had highly skewed sex ratios, and this may have been brought about by exposures to the chemical odorant. Sex ratios of the most abundant louse species (*Quadraceps aethereus*) were highly skewed towards females (185 females: 3 males) on crested auklets; however, the sex ratio of *Quadraceps* was nearly equal (29 females: 30 males) on least auklets (J. Malenke et al., unpubl. data). Female-biased sex ratios in avian fleas (Siphonaptera: Ceratophyllidae) have been attributed to male-biased mortality from chemical exposure (Shutler et al. 2003). This was attributable to smaller body size in male fleas (Shutler et al. 2003), and likewise male *Q. aethereus* are smaller than females (as is generally true for Phthirapteran chewing lice, J. Malenke, pers. comm.).

Among vertebrates the examples of chemical communication and olfaction are the least well established for the Class Aves (Agosta 1992, Roper 1999). The behavioral response of crested auklets to synthetic analogues of their odor indicates that this species could communicate with chemical signals (Chapter 8). It is not clear by what sensory modality this occurs. Experiments with synthetic odorants and heart-rate monitoring in anesthetized crested auklets failed to demonstrate an olfactory response to odor

constituents of crested or whiskered auklets (Douglas and Silver, unpubl data). Instead, individual heart rates and respiratory rates remained remarkably constant throughout these experiments. Heart rate and respiratory rate did not differ significantly after synthetic *Aethia* odorants were supplied to the nostrils.

Attributes of the chemical signaling system of crested auklets do not conform to the most restrictive definitions of the term "pheromone" (Beauchamp et al. 1976, Johnston 2000, Schaal et al. 2003). The formal criteria for what constitutes a pheromone have been articulated as follows: 1) the chemical signal is simple; 2) the behavioral response in the receiver is unambiguous, morphologically invariable and has obvious functional significance; 3) the coupling of stimulus and response is highly selective; 4) reception of the signal in the receiver is species specific; 5) coupling of the response to the stimulus is unconditional. The *Aethia* odorants appear to be species-specific in their chemical composition. However, behavioral response to synthetic analogues of whiskered and crested auklet odorant did not indicate a preference for either signal. The major odor constituents of whiskered auklets elicit at least as strong a response from wild crested auklets as does the chemical signal of conspecifics. Dodecenal isomers were not included in behavioral trials because these compounds are not available commercially. It may be that the composite signal (hexanal, octanal, decanal, z-4 decenal and dodecenals) would present a more compelling stimulus; however, the difference in response might be more a matter of degree. Crested auklet and whiskered auklet scents appear to elicit the same types of behavioral response (Chapter 8). Some other characteristics of the crested auklet odorant appear to be consistent with four criteria of pheromones, to varying

degrees. Presentation of auklet odorants solicits an anointment behavior that is also part of courtship behavior in crested auklets. Behavioral response to the odor appears to peak during early courtship. There appears to be a strong link between chemical signal and reproduction. There also appears to be a correspondence between chemical emissions and the sex hormone progesterone. The crested auklet odorant can be classified as a chemical signal involved in reproduction; however, evidence to date suggests that the odorant does not meet the strict criteria of a pheromone.

Evolution of the crested auklet odorant could have occurred through the combined influences of sexual selection and nonsexual natural selection. This apparently resulted from the augmentation of certain biochemical pathways and a higher level of organization in some epidermal tissues. One natural function of the avian integument is to help defend against pathogens through the continual replacement of the outer corneous layer (Stettenheim 2000). Secretions of the uropygial gland also have antibacterial and antifungal properties (Pugh and Evans 1970, Jacob 1978, Jacob and Ziswiler 1982) and possibly antiparasitic properties (Moyer et al. 2003). Specialized tissues in the avian integument may perform similar functions. Menon (1984) has suggested that some specialized regions of the avian epidermis have functions that are equivalent to glands even though they lack the accepted histological profile. Cells of the epidermis secrete lipids (Menon 1984), and some specialized feathers are supplied by the products of epidermal secretions (Menon 1984, Stettenheim 2000).

The major constituents of the crested auklet odorant could have been minor byproducts of fatty acid metabolism in avian dermal layers. The skin of birds contains

abundant extracellular dermal lipids that apparently originate in an adipocyte layer and diffuse into the epidermis (Stromberg et al. 1990). Stromberg et al. (1990) suggested that free fatty acids diffuse into the avian epidermis following hydrolysis. Indeed, aldehyde constituents of the crested auklet odorant are found with corresponding fatty acid methyl esters and carboxylic acids in specialized secretory feathers (Chapter 8). This indicates that the same processes could be involved as those suggested by Stromberg et al. (1990). In crested auklets the tissues may have become more highly organized and certain biochemical pathways may have been emphasized.

Enhanced biosynthesis of aldehydes in dermal layers could have been initially promoted in the skin of ancestral *Aethia* auklets as a chemical defense against microbes. Three aldehydes found in the crested auklet odorant (octanal, hexanal and decanal) are known to inhibit *Aspergillus flavus* (Wright et al. 2000) and probably other fungi and bacteria. Enhanced secretions of antimicrobial substances could have been advantageous in protecting colonial *Aethia* auklets from skin infections, particularly one like the crested auklet that fights with a sharp hooked bill.

The origins of the chemical signals in crested auklets could have developed as suggested in Table 9.1. Enhanced production of aldehydes may have increased survival of adults and contributed to higher fitness. Odor production could have been promoted through sexual selection if the chemical signal was assessed during courtship and mating. Mating preferences could have increased the amount of odor production and emphasized certain products and pathways. The chemical signals could have diverged after

populations of species became isolated. Such a divergence may have occurred in crested and whiskered auklets (Chapter 2, Douglas et al. 2004, H. Douglas, unpubl. data).

Table 9.1 Hypothetical origins for crested auklet chemical odorant

Signal Origins -- nonsexual natural selection
Secondary products of metabolism
Excess production of byproducts favored by natural selection
Signal Development & Refinement -- sexual selection
Secondary metabolites associated with "quality"
Secondary metabolites develop into a communication signal
Differentiation of specific chemicals from biochemical noise
Emphasis of specific pathways and specific chemical products
Association of chemical signal with reproduction
Divergence of chemical signal in species and populations

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